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VOLUME V

1946-1947

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*Imprimatur* John T. McNicholas, Archbishop of Cincinnati

The Nihil Obtsat and Imprimatur neither favor nor oppose opinions or theories here recorded. They are declarations that there is nothing in the Studies of the Institutum Divi Thomae contrary to faith or morals.

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VOLUME 5 1946 - 1947

# EFFECT OF PLANT EXTRACTS ON CULTURES OF *ENDAMOEBA HISTOLYTICA*

SISTER MARY PETRONELLA SCHROEDER, C.PP.S.

On the basis of results obtained in these laboratories with various plant extracts which depressed the growth of *Shigella dysenteriae* (Shiga) in chick embryo and *in vitro*[1], a study of their activity on *Endamoeba histolytica*, the protozoan causative agent of amoebic dysentery, was undertaken.

## EXPERIMENTAL

The four plants investigated were: *Psidium guajava*, *Chrysobalanus icaco*, *Tandia aculeata*, and *Hypericum fasciculatum* obtained in the Everglades, south of Lake Okeechobee, Florida, during the winter season[2]. They were gathered and prepared for the extraction by Dr. Alfred Lonsing, Rev. Gustave Brotzge, and Miss Antoinette Helmer. The tissues of the plants had been dried in an oven at 60° C. and treated with water, ether, and hydrochloric acid solution[3] as shown in Table I.

For each test, 200 mg. of the dried extract were suspended in 10 ml. of Ringer's solution buffered with M/15 dipotassium hydrogen phosphate and M/15 potassium dihydrogen phosphate (pH 7.3). The suspension was shaken well to dissolve as much of the extract as possible, although in no case did all

[1] Nutini, L. G., *et al.*: Unpublished data.

[2] Lonsing, A.: Unpublished data.

[3] Kreke, C. W., *et al.*: Unpublished data.

TABLE I. PLANT SPECIES AND TYPE OF EXTRACT PREPARED FOR TESTING OF THEIR EFFECT ON GROWTH OF E. HISTOLYTICA *in vitro*

| Extract No.* | Plant Species | Part Extracted | Nature of Extract |
|---|---|---|---|
| 1B | Psidium guajava | bark | cold water |
| 2L | Psidium guajava | residue after ether extraction of leaves | cold water |
| 3L | Psidium guajava | leaves | 1% HCl |
| 4B | Chrysobalanus icaco | bark | cold water |
| 5L | Chrysobalanus icaco | leaves | ether |
| 6L | Chrysobalanus icaco | leaves | 1% HCl |
| 7B | Randia aculeata | bark | cold water |
| 8L | Randia aculeata | residue after ether extraction of leaves | cold water |
| 9L | Randia aculeata | leaves | cold water |
| 10L | Randia aculeata | leaves | warm water |
| 11Br | Hypericum fasciculatum | branches | warm water |
| 12Br | Hypericum fasciculatum | branches | cold water |
| 13Br | Hypericum fasciculatum | branches | 1% HCl |

*B=bark; L=leaves; Br = branches.

of the material go into solution. The mixture was adjusted to pH 7.3 with normal sodium hydroxide and autoclaved at 15 pounds pressure for 20 minutes. If, after the sterilization process, the pH was less than 6 or more than 8, a second pH adjustment was made.

The culture of *Endamoeba histolytica* and the fine grade of starch powder employed in this study were kindly furnished by Dr. Donald L. Augustine of the Department of Comparative Pathology and Tropical Medicine, Harvard University. The organisms were cultured in medium suggested by Dobell and Laidlaw[4] and the culture was maintained by transfer to fresh medium every 4 to 5 days.

[4]DOBELL, C., AND LAIDLAW, P. P.: *Parasitol.* 18 (1926): 283.

Forty ml. Erlenmeyer flasks were used. Each contained 10 ml. of inspissated whole-egg-buffered Ringer's solution, to which were added 10 ml. of egg-albumin-buffered Ringer's solution and about 5 loopsful of sterile starch powder. Each experimental flask was charged with 2 ml. of the plant extract suspension. Since maximum growth of the stock culture generally occurred on the third day, the experimental flasks were inoculated with 2 ml. of a 3-day-old culture which consisted chiefly of organisms in the trophozoite stage.

Tests using each type of extract were made in duplicate. The figures in Table II represent the averages obtained from four or five counts of the organisms in a haemocytometer chamber for each experiment and are expressed as the number of organisms per milliliter.

## RESULTS AND DISCUSSION

The values for the trophozoite and cyst content of the control cultures at the end of 3 and 7 days are given in Table II.

*Psidium guajava.* All three extracts of *Psidium guajava* (extracts 1B, 2L, 3L) favored the encystation of *Endamoeba histolytica.* This was true particularly for Extracts 1B and 3L, ws was evidenced by the high cyst-trophozoite ratios 220:250 and 130:170, respectively, for the 3-day cultures. The pH of 7.0 to 7.5 observed in these and in the other cultures in which encystation was well marked is in agreement with the observation of Chang[5]. Viable cysts persisted after 10 days in the culture grown in the presence of the cold water extract of the bark of *Psidium guajava* (1B).

*Chrysobalanus icaco.* Of the three preparations of *Chrysobalanus icaco* (Extracts 4B, 5B, 6L) the cold water extract of the bark (4B) was most effective in depressing trophozoite production but the ratio of cysts to trophozoites was higher

[5]Chang, S. L.: *Am. J. Trop. Med.* 22 (1942): 471.

TABLE II. EFFECT OF PLANT EXTRACTS ON GROWTH AND ENCYSTMENT OF ENDAMOEBA HISTOLYTICA IN DOBELL AND LAIDLAW'S MEDIUM

| Extract* No. | pH | | | No. Organisms per Milliliter | | | |
|---|---|---|---|---|---|---|---|
| | Initial | 3-days | 7-days | 3-days | | 7-days | |
| | | | | troph. | cysts | troph. | cysts |
| | | | *Control* | | | | |
| | 7.2 | 7.2 | 7.0 | 520 | 0 | 20 | 1 |
| | | | *Psidium guajava* | | | | |
| 1B | 7.2 | 7.2 | 7.5 | 250 | 220 | 28 | 0 |
| 2L | 7.3 | 7.0 | 7.0 | 230 | 23 | 5 | 1 |
| 3L | 7.0 | 7.0 | 7.3 | 170 | 130 | 0 | 0 |
| | | | *Chrysobalanus icaco* | | | | |
| 4B | 7.2 | 7.0 | 7.6 | 70 | 10 | 5 | 0 |
| 5L | 7.5 | 7.1 | 7.2 | 410 | 0 | 14 | 6 |
| 6L | 7.2 | 7.0 | 7.0 | 430 | 10 | 0 | 2 |
| | | | *Randia aculeata* | | | | |
| 7B | 8.0 | 7.1 | 7.2 | 130 | 0 | 0 | 0 |
| 8L | 7.0 | 7.3 | 7.0 | 1500 | 210 | 0 | 0 |
| 9L | 7.0 | 7.4 | 7.0 | 1150 | 70 | 0 | 0 |
| 10L | 7.0 | 7.4 | 7.0 | 1000 | 130 | 0 | 0 |
| | | | *Hypericum fasciculatum* | | | | |
| 11Br | 8.0 | 7.8 | 7.5 | 0 | 0 | 0 | 0 |
| 12Br | 8.0 | 5.0 | 5.0 | 0 | 0 | 0 | 0 |
| 13Br | 8.0 | 7.2 | 7.0 | 550 | 120 | 0 | 0 |

*Extracts of bark tissue=B; leaf=L; branch=Br. See Table I for Species of Plants.

in this than in the cultures with extracts of the leaves. The organisms grown in the presence of the ether extract of the leaves (5L) remained the most viable, obtaining a 6:14 cyst-trophozoite ratio in the 7-day-old cultures.

*Randia aculeata.* At the end of the first three days the trophozoite count in the cultures charged with the cold water extract of the bark (7B) was 25 per cent that of the control culture, and there were no cysts. In contrast to this, the extracts of the leaves of this same plant (8L, 9L, 10L) stimu-

lated production of trophozoites 2 to 2.5 times that in the control cultures, with a concomitant production of cysts. After 7 days neither trophozoites nor cysts were present in any of the cultures.

*Hypericum fasciculatum.* The results obtained with the 3-day-old cultures show that the warm and cold water extracts of the branches of *Hypericum fasciculatum* (Extracts 11Br and 12Br) completely inhibited the growth of the protozoan parasite since neither trophozoites nor cysts could be detected in the cultures at that time. Results with the acid extract of the branches of *Hypericum fasciculatum* present a marked contrast, as there was an abundant formation of both trophozoites and cysts. After 7 days, however, neither trophozoites nor cysts were present in the cultures.

## SUMMARY

The effects of 13 crude extracts of 4 different plants were studied on cultures *in vitro* of *Endamoeba histolytica.* In the majority of cases the decrease in the number of trophozoites at the end of 3 days was accompanied by an increase in the number of encysted amoebae. The warm and cold water extracts of *Hypericum fasciculatum,* which were the most effective as destructive agents, caused complete inhibition of the growth of the organisms in a period of 3 days.

Original Manuscript Submitted
November 25, 1946

# EFFECT OF SOME ORGANIC METAL SALTS ON YEAST GROWTH

CORNELIUS W. KREKE AND REV. LOUIS V. NADEAU, O.P.

FROM preliminary experiments performed in this laboratory, results were obtained which indicated that some metal salts of organic acids such as citric acid were effective in accelerating the respiration of yeast cells and animal tissue slices. It seemed of interest therefore to study the effect of a series of these compounds on proliferation, using yeast as a test organism.

## EXPERIMENTAL

The assay method for measuring yeast growth and for estimating the effects of the salts was similar to that described in previous reports[1], [2], [3] with some modifications. Instead of the rocker tubes, ordinary pyrex test tubes (1.8 x 15.0 cm.) were used. These were placed in racks and shaken in an air bath maintained at 30° C. A special test tube holder was mounted on the photodensitometer so that the yeast crop could be estimated without removal of the yeast suspension from the tubes. Only test tubes giving exactly the same reading on the photodensitometer were selected for use in these experiments. This procedure, which is similar to that of Atkin, Schultz and Frey[4], has an advantage over those previously employed by us in that the yeast crop can be estimated at any time during the course of the experiment.

The yeast used was a pure strain of *Saccharomyces cerevisiae* isolated from Fleischmann's bakers' yeast and grown from a

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[1]COOK, E. S., HART, SR. M. J., AND STIMSON, SR. M. M.: *Biochem. J.* 34 (1940): 1580.

[2]LOOFBOUROW, J. R., DWYER, SR. C. M., AND CRONIN, SR. A. G.: *Biochem. J.* 35 (1941): 603.

[3]COOK, E. S., AND CRONIN, SR. A. G.: *These Studies* 3 (1941): 205.

[4]ATKIN, L., SCHULTZ, A. S., AND FREY, C. N.: *Arch. Biochem.* 1 (1943): 9.

single cell. The growth from a 3-day-old subculture on a Sabouraud's slant was suspended in Reader's medium[5], the concentration determined photometrically and adjusted to 0.78 mg. dry weight of yeast per ml. Control tubes contained 11.5 ml. of Reader's medium (pH 5.6) and 0.5 ml. of the yeast suspension to which was added 0.5 ml. of distilled water to make a total volume of 12.5 ml.

The salts tested were the citrates, tartrates, cis-aconitates, and sulfates of manganese (manganous), iron (ferric), copper (cupric), ammonium, sodium, potassium, magnesium, and calcium. Saturated solutions of the citrates and tartrates were prepared by shaking a suspension of the pure salt in water and then filtering. The concentration was determined by drying 5 ml. of the solution prior to adjusting the pH to 5.6.

The cis-aconitates were prepared by mixing equimolar concentrations of cis-aconitic acid with the metal sulfate and adjusting the pH to 5.6. Cis-aconitic acid was prepared according to the procedure of Malachowski and Maslowski[6]. The solutions of the metal sulfates and of the chloride, in the case of calcium, were prepared by dissolving the pure salt in water in concentrations equal to that used for the organic salts.

Experimental tubes were prepared in a manner similar to that for the control tubes except that 0.5 ml. of the salt solution to be tested was used instead of the 0.5 ml. of distilled water. The salts were tested for their activity on yeast growth over a range of concentrations. The tests were made in duplicate for each concentration of salt tested and the values reported are averages of the results obtained. Control tests were made in triplicate.

## RESULTS

The stimulation of yeast growth is reported in percentage increase of the yeast crop over that of the control after a

[5] Reader, V.: *Biochem. J.* **21** (1927): 901.

[6] Malachowski, R., and Maslowski, M.: *Ber.* **61 B** (1928): 2521.

TABLE I. EFFECT OF CITRATES ON YEAST GROWTH

| Metal Ion | Citrate Salt Concentration | Increase Yeast Crop over Controls | Metal Ion | Citrate Salt Concentration | Increase Yeast Crop over Controls |
|---|---|---|---|---|---|
| | μg./ml. | % | | μg./ml. | % |
| $Li^+$ | 320 | 400 | $K^+$ | 320.00 | 57 |
| | 160 | 900 | | 160.00 | 100 |
| | 80 | 780 | | 80.00 | 71 |
| | 40 | 750 | | 40.00 | 49 |
| | 20 | 740 | | 20.00 | 20 |
| $Na^+$ | 160 | 224 | $Mn^{++}$ | 320.00 | 0 insol. |
| | 80 | 170 | (as sodium | 160.00 | 0 insol. |
| | 40 | 137 | manganese | 80.00 | 0 insol. |
| | 20 | 110 | citrate) | 40.00 | 31 |
| | 10 | 0 | | 20.00 | 0 |
| $Mg^{++}$ | 1,200 | 380 | $Fe^{+++}$ | 160.00 | 112 |
| | 1,000 | 366 | | 80.00 | 61 |
| | 500 | 246 | | 40.00 | 24 |
| | 250 | 460 | | 20.00 | 12 |
| | | | | 10.00 | 6 |
| $Ca^{++}$ | 6 | 218 | $Cu^{++}$ | 1.00 | 24 |
| | 3 | 124 | | 0.50 | 29 |
| | | | | 0.25 | 9 |
| | | | | 0.13 | 22 |
| | | | | 0.07 | 9 |
| | | | $NH_4^+$ | 320.00 | 648 |
| | | | | 160.00 | 652 |
| | | | | 80.00 | 638 |
| | | | | 40.00 | 555 |
| | | | | 20.00 | 503 |

24-hour growth period. When magnesium citrate was tested for its growth stimulation activity, it was found that a precipitate formed in the medium during the growth period. For this reason it was not possible to measure the yeast crop photometrically in this case and recourse was made to its measurement by cell count*. The other citrates gave no evidence of

*Acknowledgment for these experiments is made to Sr. Ann Gertrude Cronin, S.C.

such a precipitation. When ferric citrate was tested for its effect on growth, the yeast crop was measured by both methods, photometrically and by cell count, with similar results.

Five concentration levels each for the sulfates of lithium, sodium, copper (20–320 μg/ml.), magnesium (34–540 μg/ml.), potassium (8–128 μg/ml.) and calcium chloride (40–640 μg/ml.) had no effect on the growth of yeast while the sulfate of manganese (20–320 μg/ml.) produced 92 and 37 per cent increase in growth at 20 and 40 μg/ml., respectively. Ammonium sulfate at 5 levels of concentration from 20 to 320 μg/ml. produced the highest increase of 42 per cent in the yeast crop with a concentration of 160 μg/ml.; there was no stimulation at 320 μg/ml. concentration. The ferric sulfate above 0.4 μg/ml. was insoluble, and therefore was not tested.

Of the cis-aconitates tested, only 2 salts were found to be active. The sodium salt increased the yeast crop 15 and 29 per cent at concentrations of 3 and 5 μg/ml., respectively; higher concentrations were insoluble. Ferric cis-aconitate stimulated yeast growth 70 per cent at a concentration of 4 μg/ml. but the salt was insoluble at higher concentrations. Magnesium salt (1.5–140 μg/ml., at 8 levels of concentration), calcium (6–100 μg/ml., at 5 levels), and copper (2–32 μg/ml., at 5 levels) were inactive. These same metals and the ammonium ion when tested as cis-aconitates in the same range of concentrations were also inactive.

Some of the metal ions were very active in accelerating yeast growth when tested as citrates and tartrates (Tables I and II). Among the active citrates were the lithium, sodium, magnesium, calcium, and ammonium salts. The active tartrates were the potassium and the mixed salt of potassium and sodium (Rochelle salt).

The activity of the salts is not due to the metal ion alone as shown by the inactivity of the metal sulfates, nor is it due to the citrate or tartrate ion alone since the sodium salts of

TABLE II. EFFECT OF TARTRATES ON YEAST GROWTH

| Metal Ion | Tartrate Salt Concentration | Increase Yeast Crop over Control | Metal Ion | Tartrate Salt Concentration | Increase Yeast Crop over Control |
|---|---|---|---|---|---|
| | μg./ml. | % | | μg./ml. | % |
| $Na^+$ | 320 | 13 | $Mn^{++}$ | 5.6 | 0 |
| | 160 | 23 | | 2.8 | 0 |
| | 80 | 17 | | 1.4 | 0 |
| | 40 | 9 | | 0.7 | 0 |
| | 20 | 2 | | 0.4 | 0 |
| $K^+$ | 320 | 193 | Rochelle | 320.0 | 202 |
| | 160 | 388 | Salt | 160.0 | 303 |
| | 80 | 458 | $K^+$ $Na^+$ | 80.0 | 400 |
| | 40 | 300 | | 40.0 | 278 |
| | 20 | 261 | | | |
| $Ca^{++}$ | 100 | 0 | | | |
| | 50 | 52 | | | |
| | 25 | 67 | | | |
| | 13 | 77 | | | |
| | 6 | 57 | | | |

these acids were not as active as some of the other salts; compare the values for sodium citrate with those of the other metal citrates (Table I); those for sodium tartrate with those for potassium and calcium tartrate in Table II.

## DISCUSSION

Earlier results obtained in these laboratories[7] indicate that some organic metal salts can accelerate the respiration of yeast and animal tissues, their action appearing to be concerned with the iron porphyrin enzymes of the respiratory chain.

Some salts increased the action of the enzyme catalase in the decomposition of hydrogen peroxide[8], [9], although the

[7] KREKE, C. W., AND SUTER, SR. M. ST. A.: (unpublished).

[8] KREKE, C. W., BARTLETT, SR. M. D., AND SMALT, SR. M. A.: *J. Biol. Chem.* 158 (1945): 469.

[9] KREKE, C. W., AND BARTLETT, SR. M. D.: Abstracts of papers, p. 45B A.C.S. meeting, Chicago, September 12, 1946.

metal sulfates did not do so. Since the active organic salts possessed no catalase action in the absence of the enzyme, it was concluded that they were accelerators. Insufficient information exists to determine the relationship between the enzyme acceleration and yeast growth acceleration; it is probable that some relationship does exist and it may be a close one.

From the results of this earlier work and the data presented in this paper it is apparent that metals may enter into metabolic processes in a variety of ways, the metal ions alone or metal sulfates not always being the completely active substances. Maximum acceleration of enzyme activity and of yeast growth occurred only when the metal was combined with an organic acid.

## SUMMARY

The citrate, cis-aconitate, tartrate, and sulfate salts of sodium, potassium, lithium, magnesium (++), calcium (++), manganese (++), iron (+++), and copper (++), over a wide range of concentrations, were tested on the growth of yeast cells. The ammonium salts of these acids were also tested.

All of the citrates were active. The sulfates and cis-aconitates were completely inactive at the concentration levels tested.

Some of the tartrates were only moderately active, potassium tartrate giving 458 per cent acceleration at a concentration of 80 μg/ml.

Original Manuscript Submitted
November 26, 1946

# STIMULATION OF YEAST FERMENTATION BY AN EXTRACT OF YEAST CELLS

CORNELIUS W. KREKE AND SR. ELEANOR MARIE BRADY, I.H.M.

EXTRACTS of yeast cells as prepared by Cook, Kreke, and Nutini[1], have been found to exert an accelerating effect on the aerobic metabolism of both yeast and animal tissues. Although the active principles of the extracts have not been isolated or identified, it has recently been shown that the principles exert their effect through an action on the respiratory chain of enzymes[2]. Since similar extracts depressed both the aerobic and anaerobic glycolysis of certain animal tissues[3] it seemed desirable to determine their effect on the anaerobic metabolism of yeast cells. The present paper is a report of this study. An attempt has also been made to determine the mode of action of the extract using selective poisons for the enzymes.

## EXRERIMENTAL

The procedure used for the fermentation studies was similar to that previously described[4] with several modifications. The yeast cells used for the study of anaerobic metabolism were of a pure strain of *Saccharomyces cerevisiae* subcultured from a single cell originally obtained from a cake of Fleischmann's bakers' yeast. The growth from a 3-day-old culture on a Sabouraud slant was suspended in 10 ml. of sterile Ringer's-phosphate solution containing no glucose and the concentration of cells was measured photometrically. The suspension was then diluted with additional Ringer's-phosphate solution

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[1]COOK, E. S., KREKE, C. W., AND NUTINI, L. G.: *These Studies* **2** (1938): 23.

[2]KREKE, C. W., AND SUTER, SR. M. ST. A.: *J. Biol. Chem.* **160** (1945): 105; *These Studies* **4** (1945): 85.

[3]SCHROEDER, SR. M. P., AND COOK, E. S.: *These Studies* **2** (1939): 247.

[4]NORRIS, R. J., AND KREKE, C. W.: *These Studies* **1** (1937): 137.

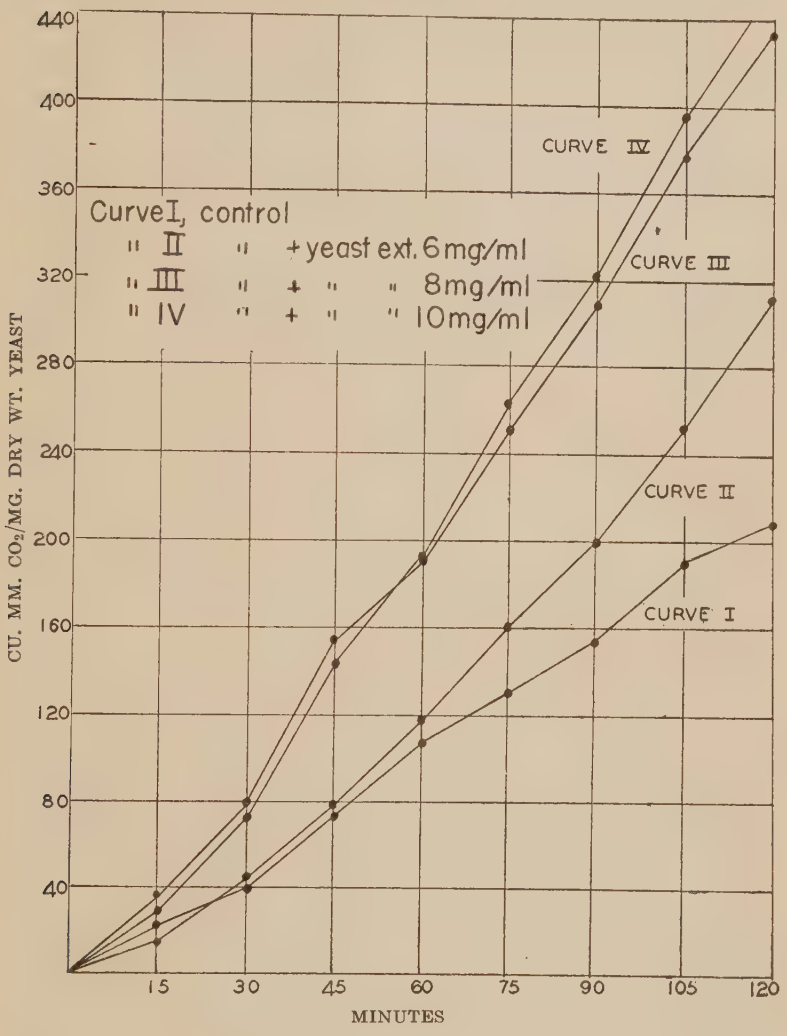
to a concentration of 1 mg. per ml. dry weight of yeast for the experiments. The concentration of yeast cells was found to give a constant rate of evolution of carbon dioxide.

Warburg respirometers were employed. The outer chamber of each flask contained a total volume of 3 ml. which consisted of 1 ml. of yeast suspension, 1 ml. of Ringer's-phosphate solution, and 1 ml. of Ringer's solution to which enough glucose had been added to make a concentration of 12 per cent, giving a final concentration of 4 per cent glucose in the respirometer flasks; the inner well contained 0.2 ml. of Ringer's-phosphate solution. This concentration of glucose supported an optimum rate of carbon dioxide evolution which remained constant throughout the experimental period. Although final concentrations of glucose as low as 0.5 per cent maintained constant rates of carbon dioxide evolution, these were below the optimum level.

The yeast extract and poisons to be tested for their effects on anaerobic metabolism were dissolved in Ringer's-phosphate solution to the desired concentration and added to the respirometer flasks in place of the Ringer's-phosphate solutions of the control flasks. The yeast extract was a commercial preparation made from Anheuser-Busch bakers' yeast corresponding to Sample A as prepared previously[1]. It was heated on a water bath for 30 minutes and filtered through a Büchner funnel. This clear syrupy solution was then adjusted to pH 7.0 with sodium hydroxide and autoclaved. Portions of this sterile yeast extract were then diluted as needed with Ringer's-phosphate solution for experimental purposes.

Each solution prior to addition to the respirometer flasks was saturated with carbon dioxide by passing the gas through it for 5 minutes and the containers were immediately stoppered to prevent loss of the gas. The solutions in the proper amounts were then placed in the flasks which were attached to the respirometers. The entire apparatus (flask and manometer

FIG. 1. EFFECT OF YEAST EXTRACT ON THE FERMENTATION OF GLUCOSE BY YEAST.




tube) was flushed for 1 minute with carbon dioxide to eliminate the air. After a 15-minute equilibrium period during which the solutions were allowed to come to temperature (30° C.) the stopcocks were closed and the carbon dioxide evolved was

measured over a 2-hour period, readings being taken every 15 minutes. Each test with the yeast extract or poison was made in duplicate and each experiment was repeated at least once.

Although preliminary experiments had shown that M/15 potassium dihydrogen phosphate containing glucose and saturated with carbon dioxide supported about the same amount of carbon dioxide evolution as did the Ringer's-phosphate-glucose solution, the rate of evolution was not constant and for this reason, the M/15 potassium dihydrogen phosphate was not used as a fermentation medium in these experiments.

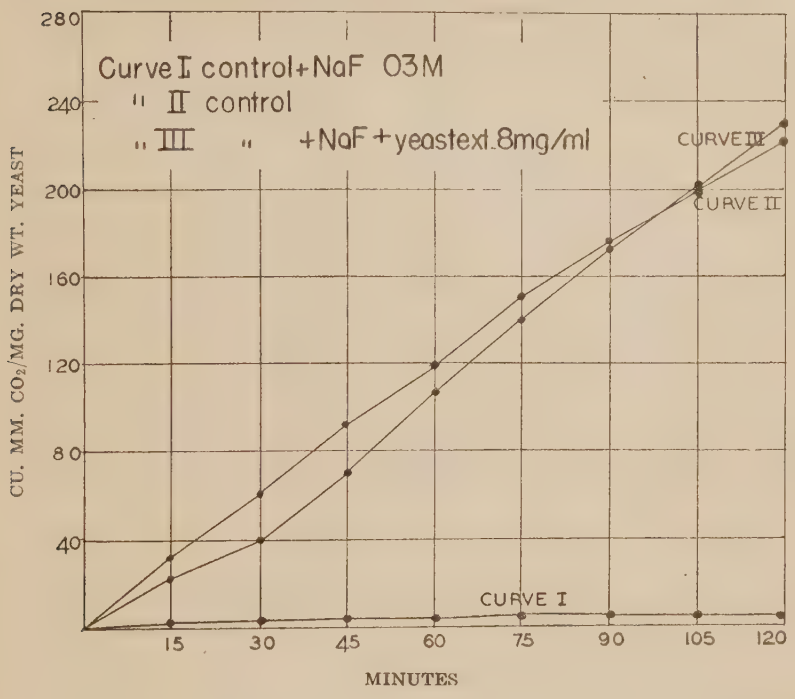
## RESULTS

There was a progressive increase in the carbon dioxide evolved with the use of increasing concentrations of yeast extract (0.5–10 mg./ml.). The optimum concentration was 8 mg. per ml. of the extract (Fig. 1, Curve III); 10 mg. per ml. gave only a very slight additional increase in the fermentation (Curve IV). The increase of carbon dioxide evolution in the presence of yeast extract is not due to the carbohydrate which the yeast extract is known to contain[5] since an optimum amount of substrate was initially present. On the other hand, other substrates more readily fermented than glucose may be present in the extract but it seems unlikely that this could account for the increased rate of carbon dioxide production.

Since sodium fluoride poisons the fermentation process by reacting with magnesium ions and binding them as magnesium fluoro-phosphate, any enzymatic process requiring these ions will be affected by this poison. This includes such enzymes as hexokinase, the phosphatases, enolase and carboxylase. Accordingly, the action of yeast extract on fermentation was

[5]Cook, E. S., Walter, E. M., Rack, F. J., Eilert, Sr. M. R., and Sawyer, M. A.: *These Studies* 3 (1941): 147.

FIG. 2. ANTAGONISTIC ACTION OF YEAST EXTRACT AND SODIUM FLUORIDE ON FERMENTATION OF GLUCOSE BY YEAST.




tested in the presence of various concentrations of sodium fluoride.

Only very small quantities of sodium fluoride are necessary to depress the fermentation of yeast, 0.03 M (final concentration), under the conditions of these experiments, being necessary to inhibit carbon dioxide formation completely (Curve I, Fig. 2). When yeast extract (8 mg. per ml.) was added at the beginning of the experiment together with the 0.03 M sodium fluoride there was complete protection against the poison, there being approximately the same rate of carbon dioxide evolution (Curve III, Fig. 2) as in the control (Curve II) in which neither yeast extract nor sodium fluoride was present.

FIG. 3. SUMMARY OF EXPERIMENTS SHOWING ANTAGONISM BETWEEN THE ACTION OF YEAST EXTRACT (8 MG./ML., FINAL CONCENTRATION) AND SODIUM FLUORIDE AT VARIOUS CONCENTRATIONS IN THE PRODUCTION OF CARBON DIOXIDE.

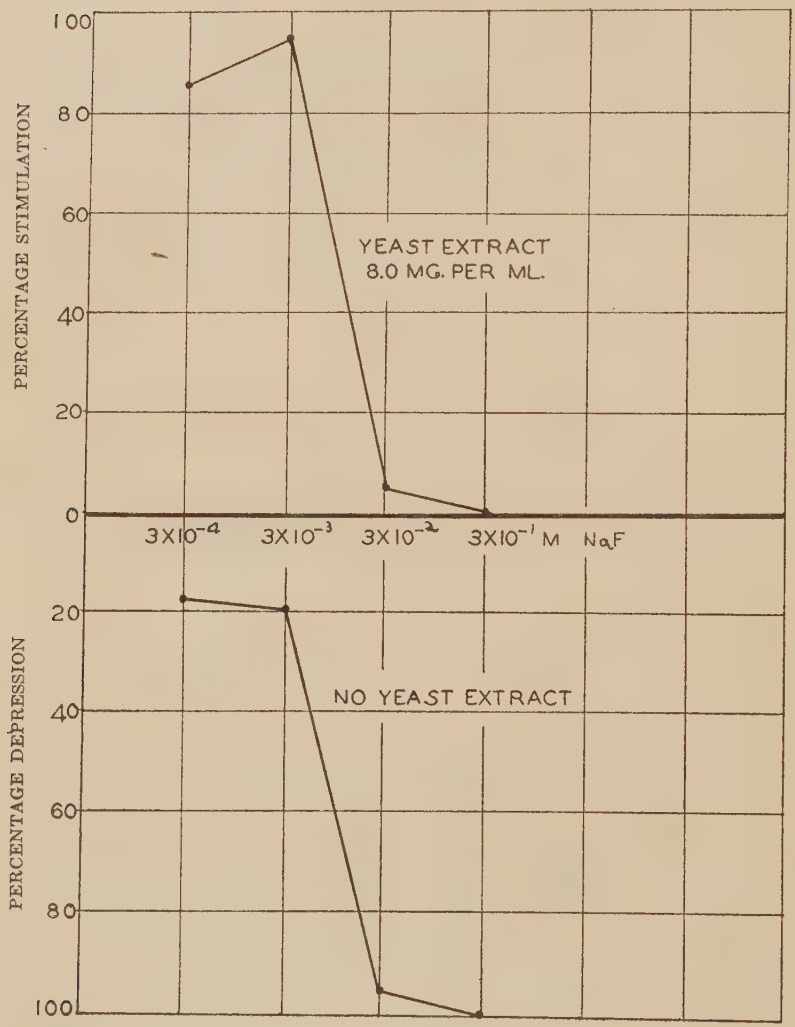
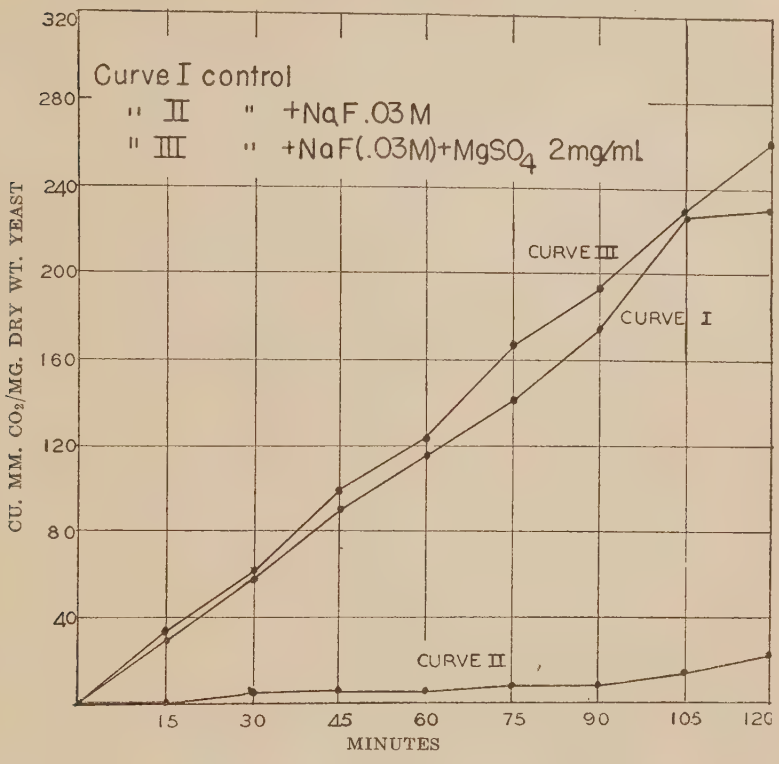
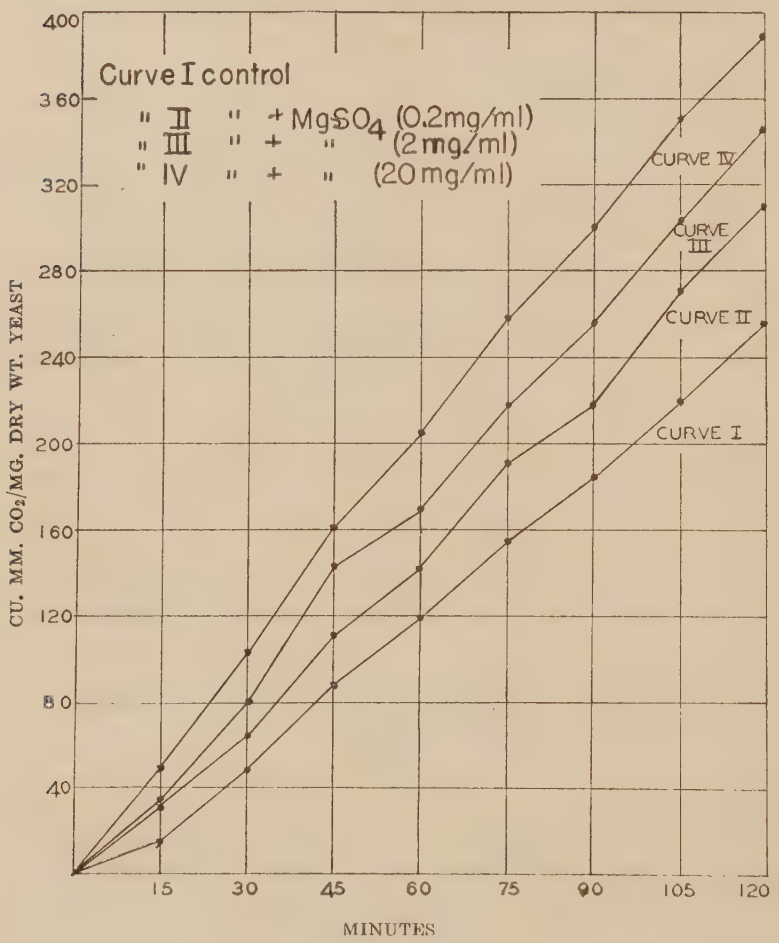


FIG. 4. ANTAGONISTIC ACTION OF MAGNESIUM SULFATE ON SODIUM FLUORIDE POISONING OF FERMENTATION OF GLUCOSE BY YEAST.



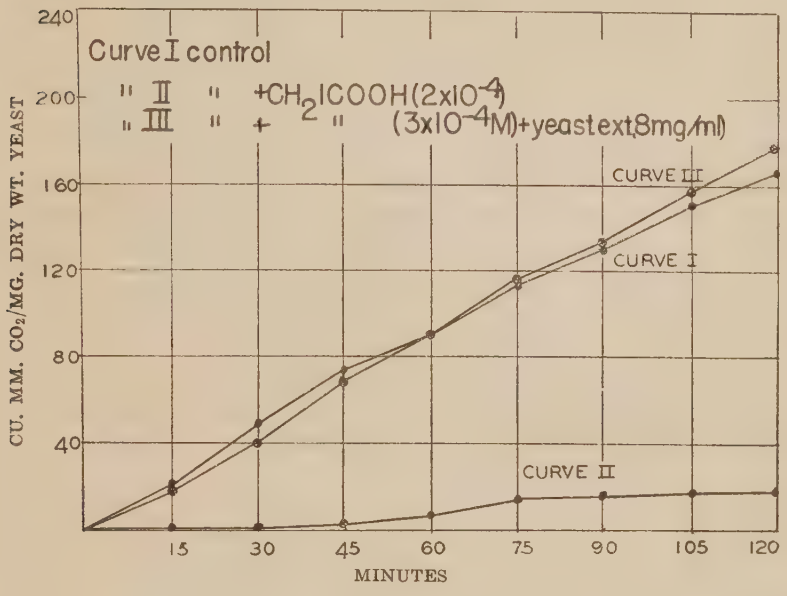

When lower concentrations of sodium fluoride were used the depression of fermentation was less than with the 0.03 M concentration and when yeast extract (8 mg. per ml. final concentration) was added with these lower concentrations of poison there was not only complete protection but an additional stimulation of the fermentation process varying inversely with the concentration of the poison present (Fig. 3). Concentrations of sodium fluoride greater than 0.3 M have not been tested but it is likely that the yeast extract would not be able to bring the carbon dioxide production back to normal in the presence of concentrations greater than this.

FIG. 5. EFFECT OF MAGNESIUM SULFATE ON THE FERMENTATION OF GLUCOSE BY YEAST.




The antagonism between the yeast extract and the sodium fluoride suggested that part of the action of the yeast extract may be due to its content of magnesium ions. Magnesium sulfate (2 mg. per ml.) effectively protected the fermentation, overcoming completely the depression due to 0.03 M sodium fluoride (Curve III, Fig. 4), and in the absence of the poison

FIG. 6. ANTAGONISTIC ACTION OF YEAST EXTRACT ON IODOACETIC ACID POISONING OF FERMENTATION OF GLUCOSE BY YEAST.




magnesium sulfate had a stimulating action on fermentation (Fig. 5). Chemical analysis of yeast extract indicates the presence of magnesium ions, but since the amount of stimulation by concentrations of magnesium sulfate as high as 20 mg. per ml. (final concentration) was not as great as that obtained with 8 mg. per ml. (final concentration) of the yeast extract (compare in Figs. 1 and 5, 73 per cent stimulation for magnesium sulfate and 94 per cent for yeast extract) it is apparent that the action of yeast extract is more complex and that only a portion of its activity is due to magnesium ions.

In a further study of the mode of action of yeast extract in stimulating fermentation, its antagonism of fermentation depression by iodoacetic acid was investigated. While this poison alkylates sulfhydryl groups[6], [7] present in many enzymes,

[6] RAPKINE, L.: *Compt. rend. Soc. biol.* **112** (1933): 1294.

[7] DICKENS, F.: *Biochem. J.* **27** (1933): 141.

such as hexokinase, phosphoglyceraldehyde oxidase, and carboxylase, it does not react with all such groups[8], and it also alkylates amino groups[9].

When yeast extract was present in the respirometer flask with the iodoacetic acid from the beginning of the experiment, there was a protection of yeast fermentation against the poison almost equal to that against sodium fluoride. In Fig. 6 is shown the ability of yeast extract (8 mg. per ml., final concentration) to overcome $3 \times 10^{-4}$ M iodoacetic acid (Curve III) which alone was enough to depress the fermentation almost 100 per cent (Curve II).

Magnesium sulfate which had shown a protective action against sodium fluoride was tested to see if it likewise would protect against iodoacetic acid. At the same concentration, (8 mg. per ml.) as that of the yeast extract, the magnesium sulfate had very little protective action as shown in Fig. 7, Curve III. From this evidence it is apparent that the protective activity of yeast extract against the action of iodoacetic acid in depressing yeast fermentation for the most part is not due to the presence of the magnesium ion.

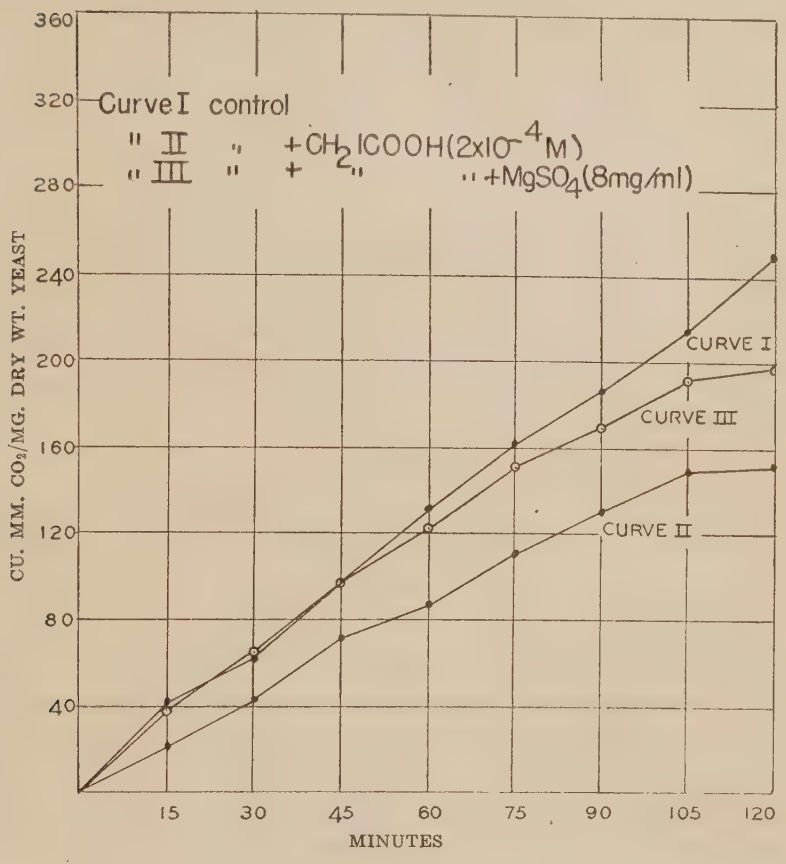
The protective action of the yeast extract probably can not be attributed to compounds containing sulfhydryl groups, which are the best known antagonists of this poison, since these have not as yet been demonstrated in the extract[5].

To determine if the action of the iodoacetic acid could be reversed once the depressant action had been established, the poison ($1 \times 10^{-4}$ M) was allowed to act on the cells for various periods of time before the addition of yeast extract. In these several experiments the yeast extract (1 ml. containing 24 mg. dry weight per ml. or enough to give a final concentration of 8 mg. per ml.) was placed in the side arm of the respirometer

[8] SMYTHE, C. V.: *J. Biol. Chem.* **114** (1936): 601.

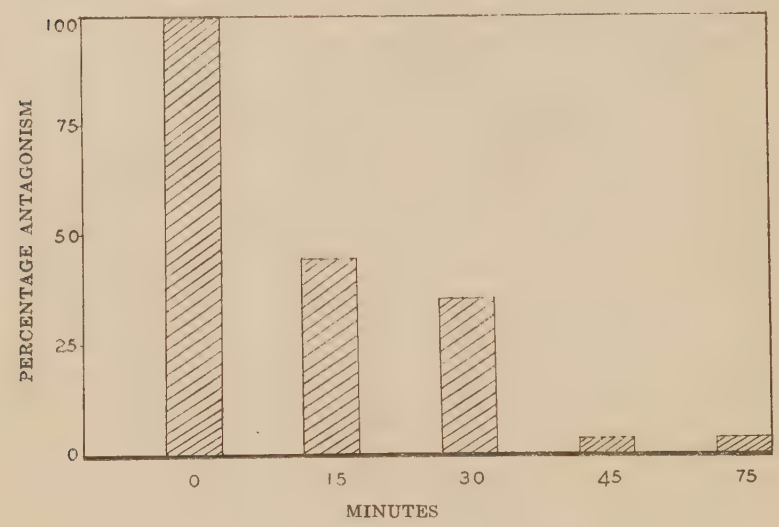
[9] BARRON, E. S. G., AND SINGER, T. P.: *J. Biol. Chem.* **157** (1945): 221.

FIG. 7. ANTAGONISTIC ACTION OF MAGNESIUM SULFATE ON IODOACETIC ACID POISONING OF FERMENTATION OF GLUCOSE BY YEAST.




flasks. When the experimental period was begun, the stopcocks of the respirometer were closed to the air and the evolution of carbon dioxide by the cells under the influence of the poison was recorded at 15-minute intervals. At the appointed time, the stopcocks were opened and the yeast extract was spilled from the side arm. A 2-minute equilibrium period was then allowed for adjustment of temperatures, before the stopcocks were closed again and the experimental period continued.

FIG. 8. ANTAGONISTIC ACTION OF YEAST EXTRACT (8 MG./ML., FINAL CONCENTRATION) ON IODOACETIC ACID ($1 \times 10^{-4}$ M., FINAL CONCENTRATION) POISONING OF FERMENTATION OF GLUCOSE, THE POISON BEING ALLOWED TO ACT FOR VARYING PERIODS OF TIME (MINUTES) PRIOR TO ADDITION OF THE YEAST EXTRACT.




The percentage antagonism by the yeast extract as shown in Fig. 8 was calculated considering as 100 per cent the depression of fermentation by the iodoacetic acid ($1 \times 10^{-4}$ M) alone. The antagonistic action which yeast extract showed for iodoacetic acid when the two were added simultaneously, was not manifested to the same degree when the poison was allowed to act for 15 minutes or longer on the cells prior to the addition of the yeast extract, the degree of antagonism being progressively less with the longer period of time the poison was allowed to act (Fig. 8). If this period of time was 45 minutes or longer before addition of the yeast extract, there was no antagonism.

## DISCUSSION

In these experiments an attempt was made to study the mode of action of yeast extracts in stimulating the fermentation

of glucose by yeast. The results obtained indicate that magnesium ions in the extract are responsible for part of the activity as shown by the fact that magnesium sulfate could antagonize sodium fluoride poisoning, but only to a small extent iodoacetic acid poisoning. In stimulating fermentation, the magnesium sulfate was only partly as effective as the yeast extract. Other active substances are therefore indicated to be present in the extract. Some results[10] have been obtained in these laboratories which indicate that certain organic salts of metals including iron and magnesium are active in stimulating respiration. Metal ions when combined with organic acids were also found to be active in accelerating the enzyme catalase[11].

## SUMMARY

Yeast extracts have been shown to accelerate the rate of yeast fermentation as measured by carbon dioxide evolution. The fermentation rate was protected from the depressing action of sodium fluoride when the yeast extract was present from the beginning of the experiment. Magnesium sulfate acted similarly in that it could also maintain the normal rate of fermentation in the presence of this poison. It was not as active as the yeast extract, however, in accelerating fermentation in the absence of the sodium fluoride.

The yeast extract could also overcome the depression caused by iodoacetic acid. The antagonism was greatest when the yeast extract was added to the fermentation medium simultaneously with the poison. If the iodoacetic acid were allowed to act on the cells for 45 minutes or longer before the extract was added, there was no antagonism.

Original Manuscript Submitted
December 1, 1946

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[10] Unpublished Data.

[11] Kreke, C. W., and Bartlett, Sr. M. D.: *J. Biol. Chem.* 158 (1945): 469.

# EFFECT OF HYDROGEN ION CONCENTRATION ON OXYGEN CONSUMPTION OF CARCINOMA 15091a FROM ABC AND dba MICE

SISTER M. VERONITA RUDDY, O.P.[1]

THE practice of determining the oxygen consumption of tissues in a physiological medium of pH 7.3 to 7.4 is open to question. With the use of vital stains, Rous[2] and his collaborators demonstrated that each organ and tissue has its individual pH which is not necessarily the same as that of the blood. By means of glass electrodes McCarrison[3] found that the pH of tissues in animals immediately after death ranged from 5.84 to 7.93. The pH of the cytoplasm of mouse mammary carcinoma 27, tar carcinoma 2146, Crocker sarcoma, and the cells in tissue culture of normal mouse mammary gland was between 6.4 and 7.0, while that of the nuclei was 7.2, as observed by Chambers and Ludford[4] after the intracellular injection of brom-cresol purple and of phenol red.

Tarantino[5], in studies of the effect of the pH of the medium on the oxygen consumption of tissues, has shown that the optimum oxygen uptake by skin, muscle and kidney of the rat occurred in media in which the pH did not in any case correspond to that of the blood. He suggested that the contradictory results reported by Kisch[6] might be due to the fact that the tissues used by Kisch were preserved for different

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[1]From the Rosary College Unit of the Institutum Divi Thomae, River Forest, Illinois.

[2]ROUS, P., AND DRURY, D. R.: *J. Exptl. Med.* 49 (1929): 435.

[3]MCCARRISON, R.: *Current Science* 1 (1932): 8.

[4]CHAMBERS, R., AND LUDFORD, R.: *Proc. Roy. Soc., London. S.B.* 110 (1932): 120.

[5]TARANTINO, C.: *Sperimentale* 91 (1937) *Sect. rend. adunanze accad. med.-fis. fiorentina*: 102. (*Chem. Abst.* 32 (1938): 7986.)

[6]KISCH, B.: *Biochem. Z.* 253 (1932): 377.

periods of time in Ringer's solution. As the pH of the medium was increased from 5.4 to 7.6 there was an increase in the oxygen consumption of mouse liver and rabbit kidney respiring in bicarbonate and in carbon dioxide-free Ringer's solution, according to Nomura[7] who also cited definite evidence that the tissues themselves acted as buffers during the experimental period.

When Comel[8] suspended muscle tissue pulp in buffered solutions at various pH values, the highest oxygen uptake was observed at a pH close to neutrality, and this diminished as a function of pH. He also found that the concentration of phosphate ion influenced the amount of oxygen consumed in media of different pH. This, he states, is probably due to the part it plays in the synthesis and cleavage of lactacidogen and the fact that the phosphate ion promotes the oxidation of the carbohydrate in the muscle.

In contrast to the reports that optimum oxygen consumption occurs at pH values near neutrality or on the acid side, Reinwein and Singer[9] working with guinea-pig tissues found the optimum pH for oxygen consumption to be 8.0 to 8.4, with considerable utilization of oxygen in media with pH values as low as 4.5 and as high as 9.6. Canzanelli[10] and his collaborators, likewise using guinea-pig tissues, observed optimum oxygen consumption by the liver, kidney and testis between pH values of 7.0 and 9.7, decreasing below pH 7.0 as a straight line function to zero at pH 4.0 on the acid side and dropping off sharply at pH 10.0 to reach zero at pH 10.5 on the

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[7]Nomura, K.: *Tohuko J. Exptl. Med.* **31** (1937): 19. (*Chem. Abst.* **31** (1937): 7502.)

[8]Comel, M.: *Boll. soc. ital. biol. sper.* **3** (1928): 550. (*Chem. Abst.* **23** (1929): 163); *Arch. Internat. Physiol.* **31** (1929): 1. (*Chem. Abst.* **24** (1930): 386); *Atti accad. Lincei* (6) **5** (1927): 307. (*Chem. Abst.* **21** (1927): 2300).

[9]Reinwein, and Singer, W.: *Biochem. Z.* **183** (1927): 315.

[10]Canzanelli, A., Greenblatt, M., Rogers, G. A., and Rapport, D.: *Am. J. Physiol.* **127** (1939): 290.

alkaline side. The optimum pH for oxygen consumption for brain tissue from the guinea pig was 9.0 to 9.5.

The present experiments on the effect of hydrogen ion concentration of the medium on the oxygen consumption of carcinoma 15091a in ABC mice were undertaken because this tumor is frequently used in laboratory studies. A chance experiment with the tumor grown in mice of the dba strain led to an investigation of possible differences in oxygen consumption values of the tumor tissues from different hosts. Also, the effect of a crude extract from yeast on the oxygen consumption of tumor tissue from ABC hosts was observed.

## EXPERIMENTAL

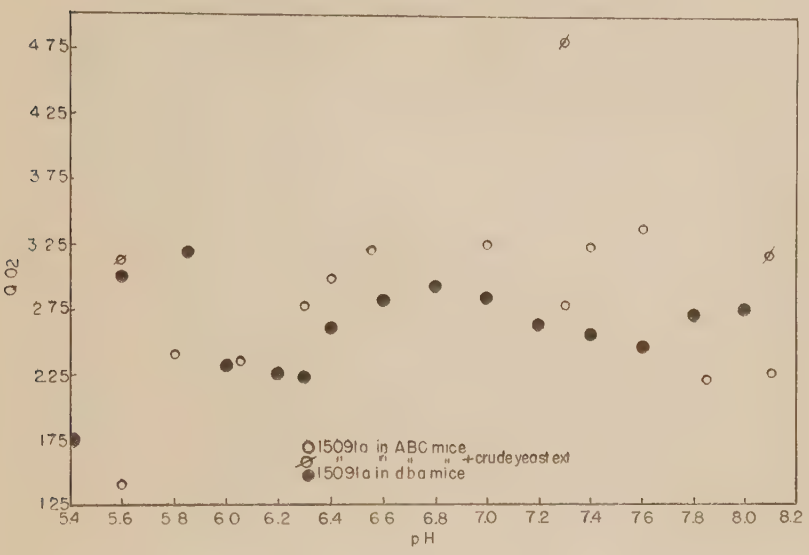
The transplantable mouse tumor 15091a used is a spindle-cell carcinoma of the mammary gland. The hosts were mice of the cancer-susceptible ABC strain and the dba strain which displays a high incidence of spontaneous mammary carcinoma. The oxygen consumption of the tissues *in vitro* was studied in air by the direct method of Warburg, and a medium of Ringer's solution and glucose buffered with a mixture of monobasic and dibasic potassium phosphate was used.

The tumor was excised from the decapitated host and placed in warm physiological saline. Small fragments of the firm portion of the tumor tissue weighing 10 to 20 mg. were put into the manometer flasks previously charged with 3 ml. of buffered Ringer's solution containing 0.02 per cent glucose. Potassium hydroxide (0.2 ml. of a 20 per cent solution) was in the inner well of each flask to absorb the carbon dioxide. Simultaneous triplicate determinations were made and readings were recorded at 15-minute intervals for an hour. At the end of this time the tissues were removed, washed in distilled water and dried at 100° C. for 16 hours. The oxygen consumption was determined on the basis of the dry weight of the tissue, as

TABLE I. OXYGEN CONSUMPTION OF TUMOR 15091a GROWN IN ABC AND dba MICE

| No. Determ. | pH Values (Avg.) initial | final | Cu. mm. $O_2$/mg./hr. avg. | range | S.D. |
|---|---|---|---|---|---|
| | | *A B C mice as hosts* | | | |
| 9 | 5.60 | 5.85 | 1.58 | (0.72–2.44) | 0.48 |
| 9 | 5.80 | 5.96 | 2.41 | (1.93–3.27) | 0.36 |
| 8 | 6.05 | 6.09 | 2.35 | (1.96–2.63) | 0.23 |
| 4 | 6.30 | 6.15 | 2.77 | (2.27–3.26) | 0.42 |
| 8 | 6.40 | 6.35 | 2.97 | (2.62–3.34) | 0.19 |
| 4 | 6.55 | 6.32 | 3.21 | (3.14–3.36) | 0.09 |
| 8 | 7.00 | 6.72 | 3.25 | (2.51–4.03) | 0.49 |
| 9 | 7.30 | 6.64 | 2.79 | (2.11–3.75) | 0.54 |
| 15 | 7.40 | 6.73 | 3.24 | (2.44–4.20) | 0.45 |
| 9 | 7.60 | 6.69 | 3.37 | (2.76–4.26) | 0.44 |
| 9 | 7.85 | 6.93 | 2.21 | (1.75–2.66) | 0.26 |
| 8 | 8.10 | 7.33 | 2.26 | (1.61–3.10) | 0.42 |
| | | *dba mice as hosts* | | | |
| 11 | 5.40 | 5.80 | 1.75 | (1.54–1.85) | 0.11 |
| 25 | 5.60 | 5.81 | 3.00 | (1.40–5.37) | 1.08 |
| 20 | 5.85 | 5.87 | 3.19 | (1.64–5.19) | 1.07 |
| 14 | 6.00 | 6.31 | 2.32 | (1.17–2.91) | 0.13 |
| 12 | 6.20 | 6.20 | 2.25 | (1.72–3.11) | 0.38 |
| 8 | 6.30 | 6.23 | 2.24 | (0.82–2.86) | 0.06 |
| 8 | 6.40 | 6.42 | 2.61 | (1.68–3.93) | 0.75 |
| 15 | 6.60 | 6.66 | 2.81 | (2.07–3.80) | 0.58 |
| 11 | 6.80 | 6.60 | 2.94 | (1.84–3.89) | 0.59 |
| 9 | 7.00 | 7.07 | 2.84 | (2.14–3.35) | 0.47 |
| 24 | 7.20 | 6.58 | 2.64 | (2.00–3.37) | 0.43 |
| 11 | 7.40 | 7.29 | 2.56 | (1.63–4.16) | 0.71 |
| 20 | 7.60 | 7.12 | 2.46 | (1.43–3.88) | 0.58 |
| 9 | 7.80 | 7.33 | 2.71 | (2.00–3.30) | 0.44 |
| 12 | 8.00 | 7.79 | 2.75 | (2.00–3.26) | 0.36 |
| | | *A B C mice as hosts—crude yeast extract in medium* | | | |
| 6 | 5.60 | 6.99 | 3.13 | (2.98–3.57) | 0.24 |
| 6 | 7.30 | 7.10 | 4.80 | (3.33–6.73) | 1.30 |
| 8 | 8.10 | 7.25 | 3.17 | (2.52–3.65) | 0.35 |

FIG. 1. $QO_2$ (CU. MM. OXYGEN PER MG. DRY WEIGHT OF TISSUE, PER HOUR) OF TUMOR 15091a IN ABC AND IN dba MICE AT VARIOUS pH VALUES




the cubic millimeters of oxygen consumed per hour by 1 mg. of dry tissue ($QO_2$).

Additional experiments were performed at pH 6.3 and 7.2 in which the number of pieces of tissue was varied in order to determine the role that this factor could play in the amount of oxygen consumed per mg. of dry tissue per hour, and in initial and final pH values.

The crude extract of yeast[11], supplied by the Institutum Divi Thomae, was adjusted to the same pH as the medium in the manometer flasks and 1 ml. of the extract was used in place of 1 ml. of the Ringer's glucose solution.

## RESULTS

The results are summarized in Table I and the average respiration data for each pH are plotted in Fig. 1. The wide

[11]COOK, E. S., KREKE, C. W., AND NUTINI, L. G.: *These Studies* 2 (1938): 23.

TABLE II. COMPARISON OF OXYGEN CONSUMPTION AND pH SHIFT OF VARIOUS NUMBERS OF PIECES OF CARCINOMA 15091a GROWN IN dba MICE

| Dry Wt. | No. Pieces of Tissue | pH Determinations initial | final | average of final | Average Oxygen Uptake | |
|---|---|---|---|---|---|---|
| *mg.* | | | | | *cu.mm./hr./mg.dry,wt.* | |
| 14.0 | 1 | 6.3 | 6.30 | | 0.87 | |
| 6.9 | 1 | 6.3 | 6.25 | 6.28±0.03 | 2.12 | 1.49±0.63 |
| 8.2 | 2 | 6.3 | 6.21 | | 2.51 | |
| 7.3 | 3 | 6.3 | 6.21 | | 2.14 | |
| 6.8 | 4 | 6.3 | 6.25 | | 2.08 | |
| 11.3 | 8 | 6.3 | 6.20 | | 2.79 | |
| 17.5 | 12 | 6.3 | 6.21 | | 2.86 | |
| 17.6 | 12 | 6.3 | 6.21 | 6.21±0.0 | 2.53 | 2.69±0.17 |
| 9.9 | 1 | 7.2 | 6.95 | | 2.58 | |
| 12.5 | 1 | 7.2 | 6.97 | | 2.00 | |
| 9.9 | 1 | 7.2 | 6.97 | | 3.05 | |
| 7.9 | 1 | 7.2 | 7.01 | 6.98±0.03 | 2.30 | 2.48±0.33 |
| 13.6 | 3 | 7.2 | 6.60 | | 2.29 | |
| 14.2 | 4 | 7.2 | 6.82 | | 2.86 | |
| 14.3 | 4 | 7.2 | 6.90 | 6.86±0.04 | 2.12 | 2.49±0.37 |
| 9.8 | 5 | 7.2 | 7.00 | | 2.71 | |
| 9.8 | 5 | 7.2 | 6.95 | 6.97±0.03 | 3.37 | 3.04±0.33 |
| 16.1 | 6 | 7.2 | 6.58 | | 3.31 | |
| 18.2 | 6 | 7.2 | 6.85 | | 3.30 | |
| 17.0 | 6 | 7.2 | 6.81 | | 2.92 | |
| 16.3 | 6 | 7.2 | 6.80 | 6.76±0.09 | 3.00 | 3.13±0.17 |

range of values for oxygen consumption in some instances is attributed to varying proportions of tumor cells and connective tissue.

Tumor tissue from ABC mice respiring in media at various hydrogen ion concentrations had the highest rate of oxygen consumption per mg. of dry tissue per hour in media at pH 6.4

to 7.6 ($Qo_2$ 2.97 to 3.37). In the dba mice the optimum $Qo_2$ was approximately 2.8 to 2.9 at pH 6.6 to 7.0.

When a crude extract of yeast was incorporated in the media at pH 5.6 for tumor tissue from ABC mice, the $Qo_2$ was increased 98 per cent. At pH 7.3 and 8.1 the $Qo_2$ was increased 63 and 24 per cent, respectively, over that of tissue without the crude extract of yeast.

Data were obtained on the final as well as the initial pH of the medium for the tumor from both the ABC mice and dba mice. The averaged final pH values for ABC mice were greater than the initial pH of 5.60 to 6.05; they were consistently lower at the end of the experimental period when the initial pH was 6.05 and higher. For the dba mice a consistently lowered final pH was not observed until the initial pH was 7.00 and greater.

As shown in Table II, the change in pH varied less at 6.3 regardless of the number of pieces of tissue, than it did at 7.2, while the number of pieces of tissue could not be correlated with pH shifts in any instance. Likewise, there is no apparent correlation in the data recorded between the number of pieces of tissue and the $Qo_2$.

## SUMMARY

The respiration of the mouse mammary carcinoma 15091a grown in ABC and dba mice was determined in Ringer's-phosphate-glucose solution at pH values ranging from 5.40 to 8.10. For the tumor grown in ABC mice, the highest rate of oxygen consumption occurred between pH 6.4 and 7.6 ($Qo_2$ 2.97 to 3.37). For the tumor grown in dba mice an optimum $Qo_2$ of approximately 2.8 to 2.9 exists at pH 6.6 to 7.0.

Original Manuscript Submitted
December 23, 1946

# EFFECT OF YEAST EXTRACTS ON CLOTTING TIME OF BLOOD

L. G. NUTINI, REV. V. J. LEWIS, SR. M. A. McDOWELL, O. P., AND A. J. BERGER

On the basis of observation that the addition of an alcoholic extract of yeast cells in low concentration to citrated blood prolonged the interval prior to disintegration of the neutrophilic leukocytes and maintained the total white count at nearly normal levels[1], the effect of extracts of yeast on blood clotting was investigated and followed by a study of the mechanisms involved.

## MATERIALS AND METHODS

A crude aqueous alcoholic extract of yeast, *S.cerevisiae*, similar to that used by Macfarlane[1] was prepared as described by Cook, Kreke and Nutini[2]. Blood was obtained from six normal subjects by the method of Todd and Sanford[3], except that a dry syringe was used. Two series of 7 experiments each were conducted. Each experiment required 6 paraffin-lined 5 ml. glass precipitation tubes, calibrated to receive 1 ml., with paraffined corks. Two tubes served as controls, 1 of which was used to determine the normal clotting time. The other contained 0.1 ml. of physiological saline when the blood was added, to determine the possible effect of dilution on clotting time. The 5 experimental tubes contained respectively 1, 3, 5, 7 and 9 per cent of the dried extract per volume of blood used. All but the 1 per cent solution were hypertonic, as shown by the cryoscopic method using the Beckmann thermometer.

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[1]MacFarlane, E. W.: *Proc. Soc. Exptl. Biol. Med.* 56 (1944): 30.

[2]Cook, E. S., Kreke, C. W., and Nutini, L. G.: *These Studies* 2 (1938): 23.

[3]Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods. W. B. Saunders Company, Philadelphia. (1935): 210.

The method for determination of the clotting time, essentially that of Lee and White as described by Stitt, Clough and Clough[4], was to add the 1 ml. of blood to the extract in the tube, which was quickly stoppered and inverted to ensure thorough mixing of the fluids before placing in the rack. The coagulation time was reckoned from the time the blood was drawn until a clot was formed which failed to flow when the tubes were inverted.

Comparisons were made of the amount of crenation occurring in the presence of the saline and of the various percentages of yeast extract, and also in the presence of 1 per cent heparin (Lederle). Tests for the effect of saline solutions of the same tonicity as that of the various yeast extracts were also made.

The yeast extract was passed through ionic exchange resins in an attempt to isolate the factor responsible for the prolongation of the clotting time and to determine the mechanism of its action, the same technique of testing clotting time being used as in the preceding experiment. The resins, obtained from Resinous Products and Chemical Company of Philadelphia were Amberlite IR-100 S.G. and Amberlite IR-4-B. S. G.

## RESULTS

From the data in Table I it is apparent that the clotting time of the control specimens varied in the different subjects and in the same subject, while the presence of the physiological saline in some instances increased and in others decreased the clotting time of the blood. In the presence of crude extracts of yeast, of 5 to 9 per cent concentrations, clotting times were

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[4]Stitt, E. R., Clough, P. W., and Clough, M. C.: Practical Bacteriology, Haematology and Animal Parasitology, Ninth Edition. The Blakiston Company, Philadelphia. (1943): 317.

TABLE I. EFFECT OF CRUDE YEAST EXTRACT ON CLOTTING TIME OF BLOOD

(*Time in Minutes*)

| Subject No. | Control | Saline 0.1 ml. | Per cent Extract in 0.1 ml. Saline | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 | 3 | 5 | 7 | 9 |
| 1 | 13.0 | 13.0 | 24.0 | 34.0 | 55.0 | 106.0 | >24 hr. |
| 2 | 15.0 | 10.0 | 12.0 | 28.0 | 52.0 | 113.0 | >24 hr. |
| 3 | 9.0 | 11.0 | 10.0 | 16.0 | 32.0 | 63.0 | >24 hr. |
| 4 | 13.0 | 9.0 | 11.0 | 17.0 | 30.0 | 102.0 | 157.0 |
| 5 | 14.0 | 13.5 | 9.5 | 16.5 | 72.5 | >24 hr. | >24 hr. |
| 6 | 9.0 | 15.0 | 9.0 | 12.0 | 12.5 | 33.0 | >24 hr. |

significantly increased and clotting failed to occur within 24 hours in 5 of the 6 specimens having 9 per cent yeast extract in them. Later experiments showed that freshly prepared extracts prevented clotting in concentrations as low as 3 per cent. Microscopic examination of the non-coagulated blood showed crenation in a high percentage of the cells. This was also found to be the case in heparinized blood, but only after 18 hours. It was felt that the crenation of the cells was due to the hypertonicity of the extract. Hypertonic saline solutions equivalent in tonicity to that of the effective yeast extracts did not affect the clotting time of the blood, indicating that the hypertonicity of the extracts was not responsible for prolongation of the clotting time.

The deionized fraction of the extract, upon testing, had no effect on blood clotting. The anions were recovered from the resins in the form of their acids, and the cations were eluted as carbonates. The activity was apparently limited to the

TABLE II. EFFECT OF YEAST EXTRACT FRACTIONS FROM THE IONIC EXCHANGE TOWERS ON BLOOD CLOTTING

| Material Tested | Clotting Time (minutes) |
|---|---|
| *Experiment A* | |
| Control | 15 |
| Control | 15 |
| Yeast extract, 10% | >24 hr. |
| Deionized yeast extract, 5% | 8 |
| Deionized yeast extract, 10% | 15 |
| *Experiment B* | |
| Yeast extract, 10% | >24 hr. |
| Yeast extract, 10% | >24 hr. |
| Anion extraction (0.2 ml.) | >24 hr. |
| Anion extraction (0.2 ml.) | >24 hr. |
| Cation extraction (0.2 ml.) | 25 |
| Cation extraction (0.2 ml.) | 17 |
| Deionized yeast extract (0.2 ml.) | 8 |
| Deionized yeast extract (0.2 ml.) | 8 |
| Control | 6 |
| Control | 5 |

anion extraction (Table II). A simple qualitative analysis of the acid groups showed the presence of carbonate, phosphate, sulfate and chloride. Since carbonates and phosphates form insoluble compounds with calcium, they can act as anticoagulants in the manner of citrates and oxalates, and render unavailable for clotting the essential calcium ions.

Original Manuscript Submitted
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# EFFECTS OF EXTRACTS OF YEAST AND MALT COMBINGS ON THE GROWTH OF, AND PENICILLIN PRODUCTION BY, *PENICILLIUM NOTATUM*

SISTER MARY PETRONELLA SCHROEDER, C.PP.S.

In view of the increasing importance of bacteria and fungi as sources of metabolic products with antibiotic activity, methods for enhancing both growth of the organisms and their production of antibiotic substances were investigated. Extracts of yeast and malt combings prepared in these laboratories have been shown to stimulate growth and respiration of microorganisms[1, 2, 3, 4, 5] and fungi[6, 7]. On the basis of these results their effect on the growth of *Penicillium notatum* in surface culture was undertaken.

## EXPERIMENTAL

*Materials.* The organism used in this work, *Penicillium notatum* NRRL 1249.B21, was kindly furnished by Dr. Kenneth Raper, of the Northern Regional Research Laboratory, Peoria, Illinois. The stock culture was maintained by the transfer of spores to partially dried Sabouraud's agar slants. The spontaneous loss of ability to produce penicillin by *P. notatum* has

[1]Cook, E. S., Kreke, C. W., and Nutini, L. G.: *These Studies* **2** (1938): 23.

[2]Loofbourow, J. R., Norris, R. J., and Morgan, Sr. M. N.: *These Studies* **1** (1937): 193; Loofbourow, J. R., and Morgan, Sr. M. N.: *J. Bact.* **39** (1940): 437.

[3]Cook, E. S., Hart, Sr. M. J., and Stimson, Sr. M. M.: *Biochem. J.* **34** (1940): 1580.

[4]Schroeder, Sr. M. P., and Hollencamp, C. H.: *These Studies* **3** (1941): 193.

[5]Schroeder, Sr. M. P.: *These Studies* **4** (1945): 67.

[6]Cook, E. S. and Kreke, C. W.: *Nature* **146** (1940): 688.

[7]Kreke, C. W.: Unpublished data.

been ably commented on by Foster, *et al.*[8] and Clutterbuck, *et al.*[9] and is due in part to the development of a mycelial type of growth. The problem of mycelial growth in the stock cultures was solved by using Sabouraud's medium in agar slants left at room temperature 3 to 4 days prior to seeding. At the end of 4 to 5 days of culture, the abundant, superficially located dark green spores could be gently washed from the slants without disturbing the mycelial growth beneath. The slants which were not used immediately were stored in the refrigerator until needed.

Three of the 5 preparations studied for effect upon growth and penicillin production were from yeast, the 4th was made from malt combings, and the 5th was corn steep liquor. *Extract A* was a commercial alcoholic extract of brewer's yeast, containing 600 mg. of solid material per ml., and corresponded to *Fraction A*, originally prepared in this laboratory[1]. *Extract B* was prepared in this laboratory from *Extract A* by the addition of acetone to 85 per cent by volume. The acetone-soluble portion was concentrated *in vacuo* to dryness and then taken up in distilled water. The solid content was 150 mg. per ml. *Extract C* consisted of the acetone-insoluble fraction in the preparation of *Extract B*. The last traces of acetone were removed by heating over a steam bath. The solid content was 640 mg. per ml. *Extract D*, corn steep liquor, was kindly furnished by Mr. J. M. Newton, of the Clinton Industries, Clinton, Iowa. Its dry weight was 650 mg. per ml.

Two types of basal media were used to test the effect of the preparations on the growth and penicillin production by

[8]Foster, J. W., Woodruff, H. B., and McDaniel, L. E.: *J. Bact.* **46** (1943): 5.

[9]Clutterbuck, P. W., Lovell, R., and Raistrick, H.: *Biochem. J.* **26** (1932): 1907.

*P. notatum.* The first, that of Moyer and Coghill[10], was prepared according to the formula:

| | |
|---|---|
| sodium nitrate | 3.000 grams |
| magnesium sulfate | 0.250 " |
| monobasic potassium phosphate | 0.500 " |
| zinc sulfate | 0.044 " |
| manganese sulfate | 0.004 " |
| glucose | 2.750 " |
| lactose | 44.000 " |
| water | 1.000 liter |

The second basal medium was a modification of that of Czapek-Dox with the following formula:

| | |
|---|---|
| sodium nitrate | 3.000 grams |
| magnesium sulfate | 0.250 " |
| monobasic potassium phosphate | 0.500 " |
| ferrous sulfate | 0.015 " |
| maltose | 44.000 " |
| water | 1.000 liter |

A third medium, a synthetic one, was prepared according to the formula of White, *et al.*[11], who found on analysis of corn steep liquor that histidine, aspartic acid, and glutamic acid were active elements of corn steep liquor in the production of penicillin. The formula for this medium was:

| | |
|---|---|
| sodium nitrate | 6.00 grams |
| magnesium sulfate | 0.50 " |
| potassium chloride | 0.50 " |
| monobasic potassium phosphate | 1.50 " |
| ferrous sulfate | 0.15 " |
| glucose | 15.00 " |
| lactose | 15.00 " |
| histidine | 0.30 " |
| aspartic acid | 0.30 " |
| glutamic acid | 4.00 " |
| water | 1.00 liter |

[10]Moyer, A. J., and Coghill, R. D.: *J. Bact.* **51** (1946): 57.

[11]White, A. G. C., Krampitz, L. O., and Werkman, C. H.: *Arch. Biochem.* **8** (1945): 303.

*Methods.* The medium in final volume of 50 ml. was dispensed in 250 ml. Erlenmeyer flasks. The experimental flasks contained basal medium to which was added either yeast extract or corn steep liquor to make a final concentration of 0.5 to 50 mg. per ml. When the combined effect of these 2 was investigated, the final concentration of the yeast extract was 20 mg. per ml. and that of the corn steep liquor was 0.5 to 50 mg. per ml. The concentration selected for the yeast preparation was based on the observation that it was optimum for stimulating respiration of *P. notatum* (unpublished data). The malt-combing extract was tested for its effect in final concentration of 45 mg. per ml. Flasks containing 50 ml. of the basal medium alone served for control cultures. The media were sterilized at 15 pounds for 20 minutes, cooled to room temperature and inoculated with 1 ml. of a suspension of spores obtained by washing each slant with 25 ml. sterile distilled water in 5 ml. portions. The cultures were incubated at 25 to 30° C. for periods varying from 6 to 9 days.

At the end of the period of incubation the molds were separated from the media by filtration. An aliquot portion of the filtrate was retained for testing penicillin activity and determining the final pH. The mats were washed with distilled water in tared Gooch filter crucibles fitted by an adaptor to a suction flask. They were then dried to constant weight in an oven at 100° C. for 24 hours. Growth was measured in terms of the dry weight of the thallus of the fungus. The pH determinations were made with the glass electrode. Penicillin production was measured by the filter paper disc method.

*Method of assay.* To determine the equivalent penicillin activity represented by zones of inhibited growth in pour plate cultures of *Staphylococcus aureus*, a standard curve was estab-

lished as follows, using a combination of the methods of Welch, *et al.*[12] and Vincent and Vincent[13].

The primary standard was crystalline sodium penicillin obtained from the Food and Drug Administration, Washington, D. C., and the test organism was *Staphylococcus aureus-H* (ATCC-152).

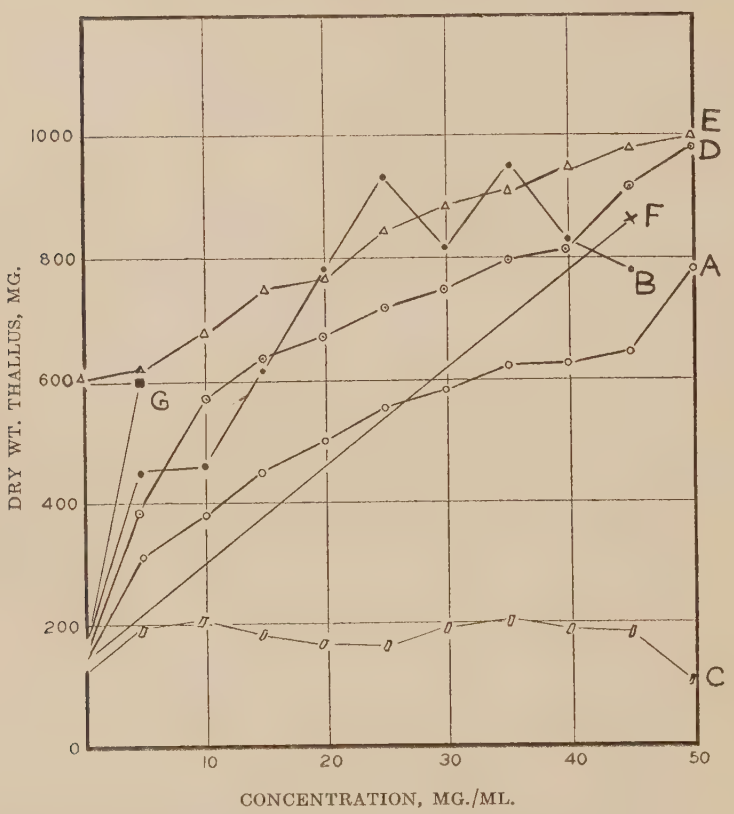
The primary standard (382 Florey units per mg.) was dissolved aseptically with pyrogen-free distilled water and 1 per cent phosphate buffer, pH 6.0, and subsequently serially diluted to contain 3.0 to 0.062 Florey units per ml.

The stock culture of the test organism was maintained on nutrient agar slants and transferred to fresh nutrient agar once a week. On the day preceding the assay, the culture of *Staphylococcus aureus* was transferred from the agar slant to peptone broth (1 per cent peptone, 0.5 per cent sodium chloride). The next day, 2 ml. of this 18 to 24-hour broth culture were added to each 100 ml. of nutrient agar which had been autoclaved and cooled to 45° C. The culture and nutrient agar were thoroughly mixed by swirling the flask and 5 ml. added to each petri plate containing a layer of 15 ml. of hardened, uninoculated nutrient agar prepared the previous day. The plates were tilted back and forth to spread the inoculated agar in an even layer over the surface. As soon as the inoculated agar layer had hardened, the plates were stored in the refrigerator until they were used in the assay, at which time 3 dry, sterilized filter paper discs (No. 470, 12.7 mm. diameter, Schleicher and Schull Co., N. Y.) were placed on each of the inoculated plates. To each disc was pipetted by means of a micro-pipette 0.1 ml. of the known dilutions of the primary standard. The plates, in an upright position, were

[12]Welch, H., Herwick, R. P., and Hunter, A. C.: *J. Am. Pharm. Assoc.*, Practical Pharmacy edition, 6 (1945): 93.

[13]Vincent, J. G., and Vincent, H. W.: *Proc. Soc. Exptl. Biol. Med.* 55 (1944): 102.

FIG. 1. EFFECT OF VARIOUS EXTRACTS ON THE GROWTH OF *Penicillium notatum* NRRL 1249.B21 HARVESTED AT 6 DAYS




A. Crude alcoholic yeast extract.
B. Acetone-insoluble fraction of A.
C. Acetone-soluble fraction of A.
D. Corn steep liquor.
E. Corn steep liquor and crude yeast extract (20 mg. per ml.)
F. Malt combings extract.
G. Histidine, aspartic acid, and glutamic acid.

immediately returned to the refrigerator until all the samples had been transferred in the same manner. The last plate employed was likewise returned to the refrigerator for about 10 to 15 minutes to allow the penicillin solution to diffuse into the discs and agar. The plates were then incubated in an inverted position for 16 to 18 hours at 37° C., since earlier work (unpublished) had shown that as soon as the antibiotic

material had been absorbed by the discs, the plates could be inverted with no difference resulting in the diameter of the inhibition zones from those incubated in an upright position. With the dilutions used, a zone of inhibition of 22 mm. corresponded to 1 Florey unit, and of 27 mm. to 2 Florey units. The portion of the curve between these 2 points was practically a straight line. When the penicillin activity of the filtrates from the experimental cultures was tested using the same technique, several dilutions of the unknown were made so that readings could be determined within this range of the curve.

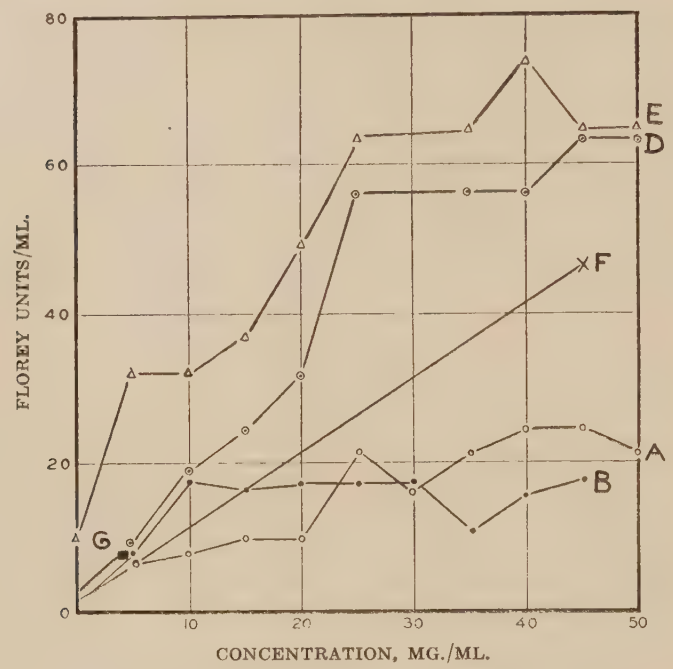
## RESULTS

With the exception of *Extract C* (Curve C, Fig. 1, acetone-soluble fraction of the crude alcoholic yeast extract) all of the materials tested stimulated the growth of *P. notatum* at the end of the 6th day. The increase in growth was proportional to the concentration used of the crude yeast extract (Curve A), the acetone-insoluble fraction of the crude yeast extract (Curve B), and the corn steep liquor (Curve D). The extract of malt combings (Curve F) and the synthetic medium (Curve G) were each tested at a single concentration. The failure of the acetone-soluble fraction of the crude alcoholic yeast extract to stimulate growth of *P. notatum* is in contrast to its stimulating effect on the growth and respiration of yeast cells[1, 3].

The highest levels of penicillin activity on the 6th day were obtained from cultures containing corn steep liquor (Curve B, Fig. 2) and corn steep liquor in combination with crude yeast extract (Curve E). The penicillin produced by the synthetic medium (Curve G) was less than that reported by White, *et al.*[11] who used the same strain of organism, but cultured it under different conditions of volume-surface ratio and used different quantities of inoculum.

The curves for growth produced by the combination of 20 mg. per ml. of crude yeast extract and varying concentra-

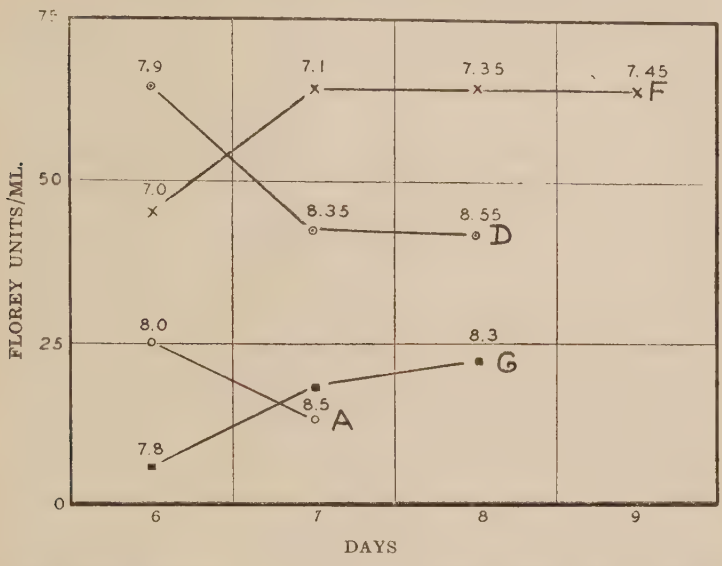
FIG 2. PENICILLIN ACTIVITY OF FILTRATES FROM 6-DAY-OLD CULTURES OF *Penicillium notatum* NRRL 1249.B21 IN MEDIA CONTAINING VARIOUS EXTRACTS




A. Crude alcoholic yeast extract.
B. Acetone-insoluble fraction of A.
D. Corn steep liquor.
E. Corn steep liquor and crude yeast extract (20 mg. per ml.)
F. Malt combings extract.
G. Histidine, aspartic acid, and glutamic acid.

tions of corn steep liquor (Curve E, Fig. 1) rose with increasing concentrations of corn steep liquor. Up to a concentration of 40 mg. per ml. of corn steep liquor and the constant 20 mg. per ml. crude yeast extract there was a similar rise in the curve for penicillin production (Curve E, Fig. 2). With higher concentrations of the corn steep liquor the yield of penicillin on the 6th day was not increased. This was associated with a high pH of the culture medium at this time. Moyer and Coghill[10] also noted a rapid rise in the pH with a high initial concentration of corn steep liquor together with a decreased yield of penicillin. The initial pH of the culture medium was

FIG. 3. RELATIONSHIP OF PH CHANGE AND PENICILLIN PRODUCTION IN VARIOUS MEDIA ON THE 6TH TO 9TH DAYS OF CULTURE




A. Crude alcoholic yeast extract.
D. Corn steep liquor.
F. Malt combings extract.
G. Histidine, aspartic acid, and glutamic acid.
pH recorded above the points on the graph.

4.5 to 5.0 and on the 6th day it ranged from 7.0 to 8.0 in the various media.

A rapid rise in pH after the 6th day was associated with a decreased yield of penicillin in the filtrates of cultures containing crude alcoholic yeast extract and corn steep liquor (Curves A and D respectively, Fig. 3). The maximum production of penicillin by the malt combings occurred on the 7th day and was sustained on the 8th and 9th days (Curve F, Fig. 3) with a concomitantly slow increase of pH from 7.1 to 7.45 during this period. The one exception to the observation of decreased yield of penicillin in presence of high pH was that of the synthetic medium (Curve G) in which the low penicillin yield on the 6th day gradually increased through the 8th day with a gradually increasing level of penicillin to 21.3 Florey units per ml.

SUMMARY

Experimental studies of methods to increase the growth of *Penicillium notatum* with a concomitant increase in penicillin yield were conducted by adding to 3 basal media a crude alcoholic yeast extract, acetone fractions of this yeast extract, corn steep liquor, malt combings extract, or the three active elements of a synthetic medium.

Growth of the mold was increased at the time of harvesting on the 6th day by all of the substances used including a combination of corn steep liquor and crude alcoholic yeast extract, with the exception of the acetone-soluble fraction of the yeast extract. When various concentrations of the materials tested were used, growth was proportionately increased with increasing concentrations. The highest penicillin production was obtained when corn steep liquor alone and in combination with the crude yeast extract was incorporated in the media.

The yield of penicillin in 7 and 8-day-old cultures was decreased in the case of crude yeast extract and corn steep liquor and was associated with a rapid increase in pH. Maximum penicillin production in the presence of malt combings was found on the 7th day and was maintained at the same level on the 8th and 9th days with little change in the pH of the medium.

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# EFFECT OF A *STAPHYLOCOCCUS AUREUS* EXTRACT ON BACTERIA

SISTER THOMAS AQUIN KELLY, O.P.,
SISTER MARGARET ANN McDOWELL, O.P.,[1] AND LEO G. NUTINI

THE present report is an extension of previous observations[2] on the effect of an alcohol-precipitated aqueous extract of *Staphylococcus aureus* on the growth and biochemistry of 4 types of bacteria so as to include the colony and cellular morphology of these and 9 additional types of bacterial organisms.

## MATERIALS AND METHODS

*Organisms tested.* The following species of bacteria were tested: *Staphylococcus aureus*[2] (the same strain as that used to produce the extract), *Escherichia coli*, *Shigella dysenteriae*, *Aerobacter aerogenes*, *Corynebacterium diphtheriae*, *Eberthella typhosa*, *Salmonella enteritidis*, *Salmonella paratyphi*, *Bacillus subtilis*, *Diplococcus pneumoniae*, Type I, *Streptococcus pyogenes*, *Streptococcus viridans* 828[3], and *Streptococcus viridans* 9811[3].

*Staphylococcus aureus alcohol-precipitated cellular extract.* The cell extract was prepared as follows: Large Roux flasks containing 250 ml. of nutrient agar were inoculated aseptically with 3 ml. of a 24-hour broth culture of *Staphylococcus aureus*, plugged, and incubated for 48 hours at 37° C. At the end of that time the bacterial cells were washed from the agar surface with 20 ml. normal saline. The washings were then frozen and thawed, and the proteins precipitated with alcohol in the same type of routine as that described for preparation of tissue

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[1]From the College of St. Mary of the Springs Unit of the Institutum Divi Thomae, Columbus, Ohio.

[2]NUTINI, L. G., KELLY, SR. T. A., AND McDOWELL, SR. M. A.: *J. Bact.* **51** (1946): 533.

[3]American Type Culture Collection.



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extracts[4]. The extract so prepared was used in 0.1, 0.5, 1.0, and 5.0 per cent concentrations in nutrient agar and in brain-heart infusion agar, the latter being used for the 3 streptococci, the *Diplococcus pneumoniae*, and the diphtheria bacillus.

*Measurement of activity.* The effectiveness of the cellular extract was measured in terms of colony counts and colony and cellular size and characteristics after 48 hours' incubation at 37° C.

The pour plate technique was used for all of the organisms with the exception of the streptococci which were streaked. For the pour plates the extract in concentrations of 0.1, 0.5, 1.0, and 5.0 per cent was incorporated in the warm agar which was then poured into sterile petri plates containing 0.5 ml. of a suspension of a 1:10,000 saline dilution of a 24-hour broth culture of the organism to be tested. The streptococci and pneumococcus were diluted 1:10 and 1:100, respectively. The control plates contained no extract.

On the basis of the colony counts for the control plates being 100 per cent, the difference between these counts and those obtained on the experimental plates was calculated. The experiments were made in triplicate.

*Colony characteristics.* Measurements in millimeters of the diameter of the surface colonies were made on the control plates and on those containing the extract in the 5 per cent concentration of the cellular extract of *Staphylococcus aureus*. Observations were made on the texture, pigmentation and type of the margin of the colony.

*Cellular characteristics.* On opposite ends of a microscopic slide smears of cells from representative colonies on both control plates and those containing cell extract in 5 per cent concentration were stained simultaneously by Gram's method. heart infusion agar, the latter medium being used for the 3 Observations on the size, grouping and general morphology of the cells were made. Some measurements of cell size were made using the micrometer eyepiece.

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[4]Nutini, L. G., Kreke, C. W., and Schroeder, Sr. M. P.: *J. Bact.* **50** (1945): 177.

TABLE I. EFFECT OF AN ALCOHOLIC CELLULAR EXTRACT OF *Staphylococcus aureus* ON THE NUMBER AND SIZE OF COLONIES OF SOME MICROORGANISMS

| Organism | % Extract Used and Colony Number | | | | Colony Diameter, mm. | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.5 | 1.0 | 5.0 | control | experimental |
| *Gram positive bacteria* | | | | | | |
| D. pneumoniae Type I | Stim. | Stim. | Stim. | Stim. | pin point | pin point |
| C. diphtheriae | Sl. Stim | Stim. | Stim. | Stim. | pin point | 0.5 |
| Strep. viridans 9811 | Sl. Stim. | Stim. | Stim. | Stim. | pin point | pin point |
| Strep. viridans 828 | Sl. Stim. | Sl. Stim. | Stim. | Stim. | pin point | pin point |
| B. subtilis | Sl. Stim. | Stim. | Stim. | Sl. Stim. | 5 | 7–8 |
| Strep.pyogenes | Sl. Inh. | Sl. Stim. | Stim. | Stim. | <1 | <1 |
| Staph. aureus | C. Inh. | C. Inh. | C. Inh. | Stim. | <1 | 2–3 |
| *Gram negative bacteria* | | | | | | |
| S. dysenteriae | C. Inh. | C. Inh. | Stim. | Stim. | 1–2 | 3–4 |
| S. paratyphi | Sl. Inh. | Sl. Stim. | Stim. | Sl. Stim. | <1 | 3–5 |
| E. coli | No Effect | Sl. Inh. | Inh. | No Effect | 2–4 | 5–6 |
| A. aerogenes | Inh. | C. Inh. | Inh. | Inh. | 1–2 | 3–3.5 |
| E. typhosa | Sl. Stim. | Stim. | Stim. | Sl. Inh. | 3 | 4 |
| S. enteritidis | Sl. Stim. | Stim. | Stim. | Sl. Inh. | no observation | |

Colony Counts: Control colony count, 100%. No effect, 75–125%.
Stimulation, 175–500%. Inhibition, 25–0%.
Slight stimulation, 125–175%. Slight inhibition, 75–25%.

## RESULTS

*Colony counts.* The effects of an alcohol-precipitated, aqueous extract of the cells of *Staphylococcus aureus* on the growth of microorganisms as determined by colony counts are shown in Table I. It is seen that all of the gram-positive organisms were

stimulated at all levels of concentration of the cellular extract used with the exception of the lowest concentration on *Streptococcus pyogenes* and the three lower concentrations on the *Staphylococcus aureus* organism itself. The effect on the number of colonies of the gram-negative organisms was variable.

*Colony size.* It is interesting to note in Table I that of the 5 gram-negative organisms for which colony size was recorded, all showed an increase in the size of the colony on plates containing 5 per cent extract whether or not there was a change in the number of colonies from that of the control. On the other hand, with the gram-positive organisms, all of which had an increased number of colonies on plates containing the 5 per cent cellular extract, there was an increase in the diameter of the colonies in only 2 instances.

*Colony texture and pigmentation.* For the most part the colonies on all of the plates, whether control or experimental, were of the same texture and pigmentation with the marked exception of the *Staphylococcus aureus* colonies which were yellow on the control and deep orange on the experimental plates.

*Colony margins.* The margins of the colonies on the experimental plates did not differ from those on the control plates with the exception of those of the gram-negative *Salmonella paratyphi.* The colonies of this organism on the plates containing the 5 per cent cellular extract had fuzzy, hazy outlines becoming contigous in many instances with those of adjacent colonies, but each remained discrete, the shape depending on the proximity of the adjacent colonies.

*Cellular characteristics.* In agreement with the observations of other investigators using extracts[4], penicillin[5, 6], and sulfanilamide[7, 8] in the study of bacteria, the individual cells in some instances appeared larger than normal, a phenomenon

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[5]FISHER, A. M.: *J. Bact.* **52** (1946): 539.

[6]GARDNER, A. D.: *Nature* **146** (1940): 837.

probably due to swelling of the cells. The cells of *Staphylococcus aureus* from plates incorporating 5 per cent cellular extract of *Staphylococcus aureus*, appeared larger by 0.1 to 0.2 microns than those from the control plate; they were more deeply stained and for the most part occurred in pairs or chains rather than in the usual cluster formation. *Corynebacterium diphtheriae* cells of the plate containing the extract were more evenly stained and were thicker than those on the control plates; no difference in grouping was noted. *Eberthella typhosa* cells from the extract-enriched agar were thicker, stained more deeply with safranin, and had a tendency to form longer chains than did those from the control plates. In the case of *Bacillus subtilis* the cells from the plate containing 5 per cent extract were smaller than the cells from the control plate and did not hold the methyl violet stain as well. There was no appreciable difference in the *Salmonella paratyphi* and *Aerobacter aerogenes* cells from the control and experimental plates. *Shigella dysenteriae* cells from plates containing the extract appeared swollen on one end and lost the methyl violet stain more readily than did the cells from control plates. The rod-shaped *E.coli* from the plate containing extract stained unevenly, some appearing to be gram positive.

## DISCUSSION

The problem of variation of pathogenic bacteria has been much in the foreground the past few years, and as Reed[9] points out, this variation has been reported most often as one of a change from the smooth to the rough form of colonies, with accompanying changes in the morphology of the individual cells. In the current experiments, although there was no change from the smooth to the rough form of colony except perhaps

[7]Lockwood, J. S.: *J. Immunol.* 35 (1938): 155.

[8]Weld, J. T., and Mitchell, L. C.: *J. Bact.* 38 (1939): 335.

[9]Reed, G. B.: Genetics of Pathogenic Organisms. Publication of the A.A.A.S. No. 12 (1940): 28.

in the case of the paratyphoid organism, there was some change in other characteristics of the colony and of the individual cells of some of the organisms studied.

It is not possible to state whether the enlargement of individual cells indicates imperfect fission or other interference with cell division as has been postulated[6]. It is well known that bacteria are not stable physiologically or morphologically when environmental conditions such as the nutrient supply is modified, giving rise in this event to numrous variants.

The results presented herein are, in general, in accordance with the findings of Nutini, Kreke and Schroeder[4] and Nutini, Hennessey and Lynch[10] in which the colon-typhoid-dysentery group reacted to extracts of beef tissues with increased colony growth and a change in the size of the organism.

SUMMARY

The effects of an alcohol-precipitated aqueous extract of the cells of *Staphylococcus aureus* were tested on 13 different bacteria. The colony counts for all of the gram-positive organisms were increased at all of the levels of extract concentration employed with the exception of the lowest extract concentration on *Streptococcus pyogenes* and the 3 lower concentrations on the *Staphylococcus aureus* organism itself. The response of the gram negative organisms was variable. In contrast, the presence of 5 per cent extract resulted in larger colonies of all of the gram-negative and 2 of the gram-positive organisms, than were found on the control plates. The presence of the extract in the agar resulted in other measurable differences in colony and cellular morphology.

Original Manuscript Submitted
March 16, 1947

[10]Nutini, L. G., Hennessey, Sr. M. E. L., and Lynch, Sr. E. M.: *These Studies* 4 (1945): 97.

# CURRENT METHODS OF PREPARATION OF EXTRACTS OF ANIMAL TISSUES FOR USE IN BACTERIOLOGIC AND CANCER INVESTIGATIONS

STAFF OF THE INSTITUTUM DIVI THOMAE[1]

In view of the increasing interest in results obtained with aqueous extracts of tissues in the study of pathogenic organisms and cancer, the current methods of preparation of small batches of these materials for use in laboratory investigations are presented in detail with notes referring to earlier and to alternate procedures.

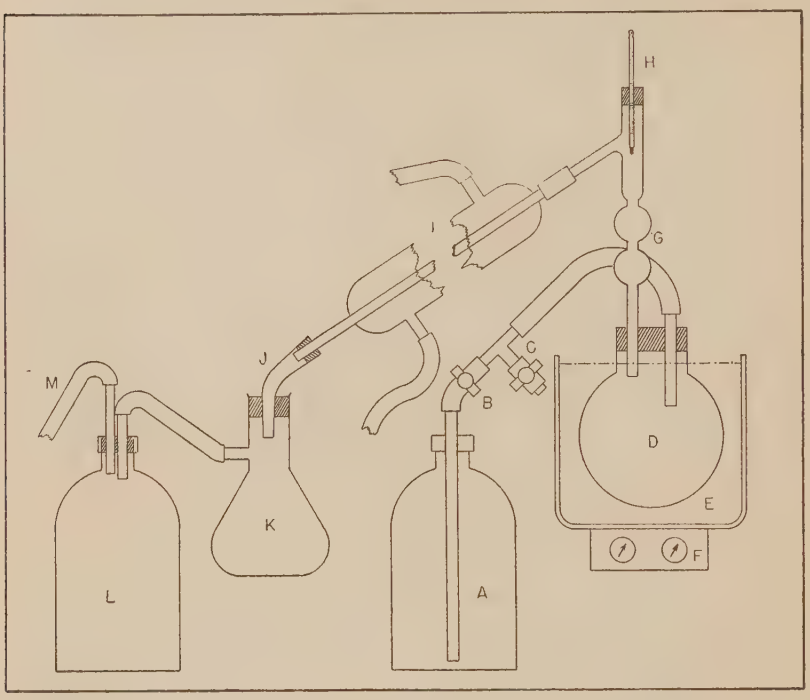
## TISSUE EXTRACTS FOR BACTERIOLOGIC INVESTIGATION

Beef spleens and brains, in lots of 3 and 10 organs respectively, and liver, heart, and kidneys in amounts of 5 to 6 pounds are obtained from a local abattoir on the day of slaughter. If necessary, they may be kept in the refrigerator 24 to 48 hours prior to use, but they should not be frozen as this results in drying of the tissue making it difficult to clean.

*Step 1. Cleaning and grinding.* All traces of blood, the pia mater or capsules, extraneous fat and larger blood vessels are removed while the organ is held under a moderately strong flow of cold tap water. The cleaned tissue is cut into pieces suitable for passing through an ordinary kitchen type of meat grinder with the medium knife blade in place. Use of the fine knife blade makes separation of tissue and fluid, as well as

[1]Through the years, modifications of the procedures for preparation of extracts have been introduced by various individuals. The techniques as described herein represent the work of L. G. Nutini, Elsie M. Walter, G. W. Thomas, E. S. Cook, G. S. Sperti, C. W. Kreke, J. C. Fardon, W. C. Abdon, and others. The present paper was compiled by Rucker Cleveland.

FIG. 1. DIAGRAM OF APPARATUS FOR CONCENTRATION OF EXTRACT IN VACUO




The reservoir (**A**) containing the alcoholic mixture is a 5-gallon bottle. A glass stopcock or a rubber tube with a screw clamp for regulating the rate of flow of fluid from the reservoir connects the outlet tube (**B**) through a T-tube (**C**) to the inlet tube in a 12-liter round bottom flask (**D**) supported in a water bath (galvanized iron bucket, (**E**) heated by an electric hotplate (**F**). The round bottom flask carries a condenser head (**G**), the upper end of which is closed by a thermometer (**H**) in a cork rather than a rubber stopper (*note* 8). To the side arm of the condenser head is attached a water-jacketed condenser (**I**), which is 4 feet in length, connected by an adapter (**J**) to a 2-liter side arm flask (**K**) which in turn is attached by a rubber hose on the side arm to a 5-gallon waste alcohol bottle (**L**); through a second tube (**M**) from the waste bottle, vacuum is obtained.

precipitation of the proteins difficult. The ground tissue is weighed.

*Step 2. Aqueous extraction by freezing and thawing.* An equal quantity of distilled water (1 ml. per gm.) is added to the

ground tissue (*note 1*). The mixture is thoroughly stirred and distributed among pans suitable for placing in the freezing chamber of a household type of refrigerator. The quick-freeze method has not been investigated for this purpose. The mixture is frozen solid once (solid freezing of the mixture is essential) and thawed, a process usually requiring 24 hours (*note 2*). The mixture is centrifuged (*note 3*) at a moderately high rate of speed (1700 r.p.m.) for 20 minutes (*note 4*), and the solid residue discarded.

*Step 3. Protein precipitation.* The protein material in the supernatant fluid (approximately 2 liters in volume) is precipitated at room temperature by adding sufficient 95 per cent undenatured ethyl alcohol over a period of 2 hours to make a concentration of 80 per cent (*note 5*). The 2 solutions are thoroughly mixed as the alcohol is added, 10.420 liters being required for each 2 liters of supernatant fluid (*note 6*). The solution is allowed to stand overnight at room temperature and the cloudy mixture is then filtered through a Büchner funnel to remove the precipitated proteins.

*Step 4. Concentration in vacuo.* The filtrate (10.172 liters) is concentrated *in vacuo* at 30-55° C. (*note 7*), a process requiring 8 to 12 hours. The apparatus designed to permit an uninterrupted process is set up as shown in Fig. 1. The level of fluid in the round bottom flask is maintained at approximately 1500 ml. by adjusting the vacuum and the screw clamp on the outlet tube from the reservoir. The large amount of free space above the liquid is useful when foaming occurs. When about one-third of the total volume of fluid remains to be distilled there may be considerable foaming. This is controlled by momentarily closing the tube (B) to the reservoir and admitting air to the circuit through the T-tube (C). The concentration process is continued until approximately 1500 ml. of solution remains in the round bottom flask, or until greasy material begins to appear on its walls. The presence of this material makes subsequent filtration difficult.

*Step 5. Filtration to remove fats.* Prior to its further concentration on the steam bath, the solution removed from the round bottom flask is cooled in the refrigerator and the congealed fatty material discarded. Passage of the solution through a non-sterile Seitz filter serves to remove more of the fatty materials. Care should be taken to regulate the vacuum during filtration to prevent foaming due to the fatty content of the solution.

*Step 6. Concentration over the steam bath.* Two methods for further concentration of the extract are in use, either one of which requires several days for completion.

*Method A.* The first method consists of the evaporation of the solution in open evaporating dishes on a steam bath by continuous addition of the solution to a final volume of approximately 500 ml. The solution may be left at room temperature overnight. When the volume of 500 ml. is obtained, it is divided into 100 ml. lots for continued concentration to 10 ml. The sides of the dishes are scraped down carefully to prevent charring. To each ml. of the cloudy brown viscuous fluid 2 ml. of distilled water is added and the solution passed through a non-sterile Seitz filter to remove the fats. Foaming should be reduced to a minimum by regulating the vacuum. The total volume of fluid is 150 ml.

*Method B.* The alternate procedure is to continue concentration of the solution in 2 large open evaporating dishes on a steam bath, the solution being added continuously during the process. As soon as the fluid in the 2 dishes is sufficiently reduced in volume, the 2 are combined in 1 dish and evaporation is continued to a final volume of 100 ml. Care is taken to prevent charring in the latter stages. The solid content per batch of the material prepared in this way is less than with *Method A.*

*Step 7. pH adjustment and sterilization.* The solutions, whether prepared by *Methods A* or *B*, are cooled, the pH

adjusted to 7.0-7.1 with 10 N sodium hydroxide or concentrated hydrochloric acid using the glass electrode. The solution is then sterilized by passage through a sterile Seitz filter, precautions being taken to prevent foaming. It is then put into sterile rubber-capped 30 ml. vials which may be stored in the refrigerator, although this latter precaution is not essential.

*Step 8. Determination of solid content.* An aliquot portion of the final solution is pipetted to a tared watch glass and evaporated to dryness in an oven at 50 to 80° C. overnight. When *Method A* is used, the solid content varies from 100 to 150 mg. per ml. for the total 150 ml. When *Method B* is used, the range is from 125 to 175 mg. per ml. for the 100 ml. of solution.

For bacteriologic investigation in mice the extract is used without further dilution, the dose in milligrams being given on the basis of the solid content per ml. The check for adequate removal of protein is a negative biuret test.

NOTES FOR PREPARATION OF TISSUE EXTRACTS FOR BACTERIOLOGIC WORK

1. Drew's solution was originally used for extraction of the ground tissue. It is a modification of Locke's solution, the preparation of which is described by A. H. Drew in the *Brit. J. Exptl. Path.* 3: 22-27, 1922.

2. Originally, 3 separate freezing and thawing processes were used, requiring 3 days.

3. In some instances the mixture was not centrifuged, the alcohol precipitation of the protein being done in the presence of the ground tissue.

4. The 20-minute period for centrifugation may be reduced to 5 to 10 minutes. The volume of the supernatant fluid is slightly reduced thereby.

5. Originally, the protein precipitation was done in three successive concentrations of 50, 70, and 80 per cent alcohol for 24 hours at each concentration, and at room temperature. For 2 liters of supernatant fluid 2105 ml. of 95 per cent undenatured ethyl alcohol is required for 50 per cent concentration; an additional 2525 ml. for 70 per cent concentration (total volume 6.73 liters) and an additional 3442 ml. for 80 per cent concentration (total volume of mixture 10.172 liters).

6. If the proteins are precipitated directly from the tissue and water mixture no correction is made for the use of 95 rather than 100 per cent alcohol, since the solid content would represent at least 5 per cent of the volume. The

volume of alcohol used in this case would be 4 times that of the original ground tissue and added distilled water.

7. Originally the temperature was maintained at 45° C.

8. A rubber stopper does not stand up as well as a cork and it and the thermometer may be pulled into the round bottom flask with sufficient force to break the latter.

### TISSUE EXTRACTS FOR CANCER INVESTIGATION

The chief difference in the preparation of the tissue extracts for cancer and for bacteriologic investigation is the use of a solvent to remove fats from extracts to be used in the study of cancer. The procedures as outlined below are those employed by Dr. Elsie Walter for use in clinical studies. Alternate procedures which have been employed are presented in the notes.

The spleens in lots of 3 to 5 organs, and liver in amounts of 4 to 6 pounds are obtained from a local abattoir. The organs are generally processed immediately, or if that is not possible, with no more than 2-3 hours of refrigeration prior to use (*note 1*).

*Step 1. Cleaning and grinding.* The tissue is weighed after thoroughly removing fat, connective tissue, and blood vessels (*note 2*), and is then cut into pieces suitable for putting through an ordinary kitchen type of meat grinder, using the finest size of knife blade (*note 3*).

*Step 2. Aqueous extraction by freezing and thawing.* An equal volume of distilled water (*note 4*) is added to the ground tissue (100 ml. per 100 gm.). The mixture is thoroughly stirred, frozen solid in a household type of refrigerator and thawed 3 times, each time at room temperature, 24 hours usually being required for each of the freezing and thawing processes (*note 5*). The total volume of the mixture is determined (*note 6*).

*Step 3. Protein precipitation.* To precipitate the protein material, undenatured 95 per cent ethyl alcohol is added over a period of 2 to 4 hours until an amount 4 times that of the

volume of tissue and water has been added (*note 7*). The mixture is thoroughly agitated after the addition of each lot of the alcohol to ensure thorough mixing of the solutions. No attempt is made to correct for the use of 95 rather than 100 per cent alcohol as the solids account for at least 5 per cent of the volume. The tissue, water and alcohol mixture is allowed to stand overnight at room temperature. The precipitated proteins and tissue pulp are removed using several Büchner funnels simultaneously. The residue is discarded.

*Step 4. Concentration in vacuo.* The filtrate, of more than 10 liters volume is concentrated *in vacuo* at 30-55° C. to one-half the volume of distilled water added to the ground tissue, a process requiring 3 to 5 days, depending on the volume of fluid and the size of the distilling flask (*note 8*). An apparatus permitting an uninterrupted process is set up in much the same manner as that described for the preparation of tissue extracts for bacteriologic work. The reservoir containing the alcoholic mixture is a 5-gallon bottle with a 2-hole stopper carrying an open air tube and a right-angled outlet tube. A glass stopcock is used for regulating the flow of the alcoholic extract into the distilling flask, short pieces of sulfur-free rubber tubing being used for making these and all connections in the distilling apparatus, and glass tubing being used wherever possible. The round bottom distilling flask (3 liter capacity) is supported in a water bath, an enamel pot, heated by an electric hotplate. To the side arm of the condenser head is attached a water-jacketed condenser, 2 feet in length, connected by an adapter to a 2-liter side arm flask which in turn is attached to a vacuum pump.

The level of the fluid in the round bottom flask is maintained at approximately 1500 ml. by adjusting the vacuum and/or the stopcock between the flask and the reservoir. If excessive bubbling occurs it may be controlled by admitting air to the circuit.

*Step 5. Ether extraction of fats and subsequent concentration in vacuo.* When the volume of the fluid is about one-half that of the original distilled water added, the solution is removed, cooled to room temperature (*note 9*) and fat is extracted with an equal volume of diethyl ether in at least 3 and possibly 4 portions. A volume of 250 ml. of ether, or one-half that of the concentrate, is added to the extract in a separatory funnel, mixed well, and the ether allowed to separate. The extract is removed and the process is repeated successively using 125 ml. portions until the ether is colorless. An hour or two is required for the entire procedure (*note 10*). The fats are easily removed when the extract is at this concentration, and completion of the concentration *in vacuo* is possible since there is practically no bubbling after the fat is removed. The concentration is continued *in vacuo* to a final volume of 30-50 ml. with no danger of charring.

*Step 6. pH adjustment and sterilization.* The volume of the concentrated extract is adjusted at room temperature with distilled water to 1 ml. equivalent to 15 gm. of the original tissue, *i.e.*, for each 15 gm. of original tissue there is 1 ml. of extract (*note 11*). The pH is adjusted to 6.8 to 7.0 with a glass electrode and concentrated sodium hydroxide or hydrochloric acid, effort being made not to go beyond pH 7.0 (*note 12*). The solution is then placed in a boiling water bath for 20 minutes, cooled, and placed in the refrigerator overnight. The next day the precipitated proteins are removed while the solution is still cold, several simultaneous filters with fluted filter paper being set up for this purpose. The volume of the solution is again adjusted to 1 ml. equivalent to 15 gm. of tissue, since some loss in volume may have occurred during the interval of heating in the boiling water bath. The solid content of this extract is approximately 220 mg./ml. The solution is then placed in 15 ml. serum bottles with rubber caps, the air exhausted with a syringe, and the bottles placed in boiling water for 20 minutes on 3 successive days. They are

kept in the refrigerator in the interim. The vials are stored in the refrigerator.

*Step 7. Tests for sensitivity.* As a check on toxicity, each batch of extract is tested by injecting 1 ml. subcutaneously into a guinea pig (*note 14*). The amount of extract used per dose intradermally for skin lesions varies with the site, which of course influences the amount of infiltration possible. Intramuscular injections are used for the more extensive types of malignancy.

NOTES FOR PREPARATION OF TISSUE EXTRACTS FOR CANCER INVESTIGATION

1. Upon occasion, the organs have been stored in the refrigerator, but not frozen, for 24 to 48 hours prior to use.

2. An alternate procedure is to weigh the tissue after it is ground.

3. Some workers with the procedure have found the medium knife blade better for grinding the tissue because of difficulty in separating tissue and fluid by centrifugation and in precipitation of the proteins when the fine blade is used.

4. Originally, the ground tissue was extracted with Drew's solution, a modification of Locke's solution described by A. H. Drew in *Brit. J. Exper. Path.* 3: 22-27, 1922.

5. An alternate procedure is to freeze the tissue and water mixture solid once, rather than 3 times, and thaw it at room temperature.

6. An alternate procedure is to centrifuge the mixture at a moderate rate of speed for 20 minutes immediately after thawing, to remove the tissue pulp. It is thought that filtration to remove the precipitated proteins in the next step is facilitated if the pulp is removed first. More recently, the period of centrifuging has been shortened to 5 to 10 minutes at a speed of 1700 r.p.m. The yield of supernatant fluid is less with the shorter period of spinning.

7. If the procedure described in *note* 6 has been followed, the volume of supernatant fluid is determined and the proteins precipitated by the addition of sufficient 95 per cent undenatured ethyl alcohol (10.42 liters for 2 liters of supernatant fluid) over a 2-hour period to bring the concentration to 80 per cent. Originally, the alcohol precipitation was done in two steps, the concentration being raised to 50 per cent for 2 hours, then to 80 per cent and allowed to stand at room temperature for 12 hours.

8. Use of a 12-liter round bottom flask in a galvanized iron bucket water bath, a 4-foot water-jacketed condenser and a 5-gallon waste alcohol bottle between the vacuum and 2-liter side-arm flask permits completion of the process within 8 to 12 hours.

9. An alternate procedure from this point is to remove the congealed fatty material and to continue concentration of the solution in open evaporating dishes over the steam bath, leaving the fat extraction with ether until later.

Several days are required to bring the volume of the concentrate to approximately 150 ml. When the process is interrupted overnight the solution is placed in the refrigerator. In this case the fatty layer in the bottom of the dish is not disturbed and it disappears on re-heating. Care must be taken to prevent charring if this technique is used.

10. Ether extraction of the 150 ml. concentrate obtained by the procedure in *note* 9 is done with an equal volume of diethyl ether divided into at least 3 separate portions. The thoroughly mixed ether and concentrated solution are left at room temperature overnight in a separatory funnel. The first ether is discarded and a second portion added, mixed thoroughly, left for 6 hours at room temperature and then discarded. The third portion of ether is added to the solution in an Erlenmeyer flask and thoroughly mixed. The flask is closed with a cotton plug and placed in the refrigerator overnight. The final ether should be colorless. The ether is discarded, and that remaining in solution removed by further concentration over the water bath in open evaporating dishes. Less ether is required if ether extraction of the fat is done with the final concentrate. On the other hand, removal of the fat from the partial concentrate is accomplished in a shorter time.

11. In earlier work a concentration of 1 ml. equivalent to 1 gm. of tissue was made; this was later increased to 9 and then to 15 gm.

12. If the procedures in *notes 9* and *10* have been followed, the solution is passed through an unsterile Seitz filter to remove additional precipitated material. In doing this there may be some foaming which can be controlled by reducing the vacuum. The filtrate is placed in the refrigerator for 12 hours and the precipitate is discarded. It is at this point that the pH is adjusted to 7.0–7.05 with a glass electrode, using sodium hydroxide or concentrated hydrochloric acid. The procedure in *note 13* is then followed.

13. At this point the solution may be sterilized by passage through a sterile Seitz filter for which process the receiving flask, filter holder, and filter are chilled until slightly frosted since further precipitation may occur when the chilled solution comes into contact with the warm glassware and filter. Due to passage through the Seitz filters the volume of the solution may be reduced until the concentration equivalent may be as high as 20 gm. of tissue per ml. The sterile solution is put into sterile 30 ml. serum bottles with rubber caps which are sealed to the glass on the outside with Duco cement. The vials are stored in the refrigerator.

14. Before initiating treatment of a patient for cutaneous malignancy or for more extensive types of it, skin tests should be made. Intradermal injection of 0.02 ml. of a 1:100 dilution of the extract (1 ml. equivalent to 15 gm. of tissue) is made and the reaction read 20 to 30 minutes later. If it is negative to moderate in degree, 0.5 ml. of the original extract is given for the first dose, and the subsequent doses are increased by 0.5 ml. of the original extract until a total of 2 ml. is given per injection. If the reaction is strong, the dose is started at 0.2 ml. and increased by 0.2 ml. each time until the maximum of 2 ml. is given.

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# ANTIBIOTIC MATERIALS FROM THE THALLI OF MOLDS

SISTER M. PETRONELLA SCHROEDER, C.PP.S., REV. ROBERT L. HAGEDORN, AND SISTER ALMA MARIE MESSING, O.P.

SUBSTANCES derived from fungi and having bacteriostatic or bactericidal properties have generally been metabolic products of molds and as such have been obtained from the medium in which the molds were cultured[1, 2, 3, 4]. In the course of the present investigations on the antibiotic properties of the water, ether, and alcohol extracts of the thalli of Aspergillus and Penicillium genera of molds, reports of similar investigations on *Penicillium notatum* by Taylor[5] were found, and the results obtained with the same mold and several additional ones were published by Taorimina[6].

## EXPERIMENTAL

*Materials.* Five of the 7 species of Aspergillus[7] and the 2 Penicillium cultures[8] used were secured from the Northern Regional Research Laboratory through the courtesy of Dr. Kenneth Raper. The 2 species, *Aspergillus glaucus* and *Aspergillus niger* (ARA) were from the stock cultures in this laboratory.

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[1] FLEMING, A.: *Brit. Med. J.* **2** (1941): 386.

[2] BUSH, M. T., AND GOTH, A.: *J. Pharm. and Exptl. Therap.* **78** (1943): 164.

[3] MENZEL, A. E. O., WINTERSTEINER, O., AND HOOGERHEIDE, J. C.: *J. Biol. Chem.* **152** (1944): 419.

[4] JONES, H., RAKE, G., AND HAMRE, D. M.: *J. Bact.* **45** (1943): 461.

[5] TAYLOR, H. G.: *Proc. Soc. Exptl. Biol. Med.* **52** (1943): 299.

[6] TAORIMINA, A.: *Boll. soc. ital. biol. sper.* **22** (1946): 589.

[7] *Aspergillus niger, Aspergillus nidulans, Aspergillus flavipes, Aspergillus flavus, Aspergillus fumigatus.*

[8] *Penicillium notatum, Penicillium chrysogenum.*

The culture medium was a modified Czapek-Dox[9] supplemented with malt combings extract, the solid content of which ranged from 60 to 70 mg. per ml.

*Methods.* For each series the medium was dispensed in 200 ml. quantities into 6 Roux flasks and sterilized at 15 pounds for 20 minutes. After cooling to room temperature each flask was inoculated with 4 ml. of a suspension of spores, prepared by combining the 10 ml. portions of washings from several 4 to 5-day-old cultures on Sabouraud's agar slants. The cultures in the Roux flasks were then incubated at 25 to 30° C. for periods of 6, 9, and 14 days, at which time the mats from groups of 2 Roux flasks were separated from the medium, washed in several portions of distilled water and partially dried by suction through a Büchner funnel. The mats when minced weighed approximately 40 to 50 gm., which amount was used for each extraction process with water, ether, or ethyl alcohol.

*Water extract.* The minced thalli were infused with 30 ml. of distilled water at 50° C. for 20 minutes, filtered, the pH of the filtrate adjusted to 7.2 and passed through a sterile Seitz filter. The resulting volume was usually about 15 ml.

*Ether extract.* Two hundred ml. of ethyl ether was added to the minced thalli in an Erlenmeyer flask and left to stand at room temperature for 24 hours and shaken occasionally. At the end of this time the ether was filtered off and the filtrate evaporated *in vacuo*. The concentrate was taken up in 10 to 15 ml. distilled water at a pH 7-8, and the solution passed through a sterile Seitz filter.

*Alcohol extract.* To the minced thalli in a shallow pan an equal weight of water was added and the mixture frozen and thawed. Ethyl alcohol was added to the thawed mixture to make 50 per cent concentration. This infusion stood for 24 hours at room temperature after which the alcohol content

[9] Hobby, G. L., Meyer, K., and Chaffee, E.: *Proc. Soc. Exptl. Biol. Med.* 50 (1942): 277.

TABLE I. ANTIBIOTIC EFFECT OF AN ALCOHOL EXTRACT OF THE THALLI OF *Penicillium notatum* AND *Penicillium chrysogenum* ON *Staphylococcus aureus-H* AS TESTED BY FILTER PAPER DISC METHOD

| Age of Culture From Which Extract Prepared | Diameter of Inhibition Zones | |
|---|---|---|
| | *P. notatum* | *P. chrysogenum* |
| *days* | *mm.* | *mm.* |
| 6 | 26 | 27 |
| 9 | 27 | 32 |
| 14 | 24 | 29 |

Diameter of disc containing antibiotic 12.7 mm.

was increased to 70 per cent by the addition of 95 per cent alcohol. The mixture again stood at room temperature for 24 hours. Since little of the material of the thalli seemed to be in solution at the time the mixture was then refluxed at 50 to 60° C. for 4 hours. The alcoholic extract was then filtered off and concentrated *in vacuo*. Final evaporation of the last traces of alcohol was done in an evaporating dish over a steam bath. During this process the temperature of the mixture rose as high as 60° C. The concentrate, about 15 to 20 ml. in volume, was adjusted to pH 7.0 and passed through a sterile Seitz filter.

*Method of assay.* The filter-paper-disc method of Vincent and Vincent[10] and Welch *et al.*[11], with modifications by Schroeder[12], was used to determine the antibiotic activity of the extracts prepared. This activity was expressed in terms of the diameter of the inhibition zones formed in pour plates of *Staphylococcus aureus-H*.

## RESULTS

None of the Aspergillus molds produced antibiotic materials by the extraction processes used on the minced thalli. The

[10] VINCENT, J. G., AND VINCENT, H. W.: *Proc. Soc. Exptl. Biol. Med.* 55 (1944): 162.

[11] WELCH, H., HERWICK, R. P., AND HUNTER, A. C.: *J. Am. Pharm. Assoc.* Practical Pharmacy Ed. 6 (1945): 93.

TABLE II. STABILITY OF ANTIBIOTIC FROM THALLI OF 6, 9, AND 14-DAY-OLD CULTURES OF *Penicillium chrysogenum* STORED AT 0° C. AND TESTED ON *Staphylococcus aureus-H* BY THE FILTER PAPER DISC METHOD

| Storage Period | Diameter of Inhibition Zones | | |
|---|---|---|---|
| | 6-day cultures | 9-day cultures | 14-day cultures |
| *days* | *mm.* | *mm.* | *mm.* |
| 0 | 27 | 32 | 29 |
| 1 | 20 | 27 | 25 |
| 2 | 16 | 25 | 22 |
| 3 | 16 | 25 | 21 |
| 4 | | 23 | 21 |
| 5 | | 23 | 21 |
| 6 | | | 21 |

Diameter of disc containing antibiotic 12.7 mm.

alcohol extracts of *Penicillium notatum* and *Penicillium chrysogenum* contained antibiotic material (Table I), the yield from *Penicillium chrysogenum* being greater than that from *Penicillium notatum*. The most potent preparations in both instances were from the 9-day-old cultures. This finding for *Penicillium notatum* differs from that of Taylor[5] who reported the greatest antibiotic activity in alcoholic extracts of thalli from 5 to 7-day-old cultures.

Over a period of weeks of storage in the refrigerator, there was a decrease in the antibiotic activity of alcoholic extracts of the thalli of *Penicillium chrysogenum* from 6, 9, and 14-day-old cultures (Table II). Activity of the antibiotic from the thalli from 6-day-old cultures was practically zero after 3 weeks' storage, while that of extracts prepared from the thalli removed from 9 and 14-day-old cultures was still present but somewhat diminished after storage at 0° C. for 6 weeks.

The antibiotic activity of alcoholic extracts of the thalli of *Penicillium chrysogenum* removed from a 9-day-old culture

[12]SCHROEDER, SR. M. P.; *These Studies*: 5 (1946): 39.

TABLE III. STABILITY OF ANTIBIOTIC FROM THALLI OF *Penicillium chrysogenum* TO A TEMPERATURE OF 100° C. AS TESTED ON *Staphylococcus aureus-H* BY THE FILTER PAPER DISC METHOD

| Time Exposed | Diameter of Inhibition Zones |
|---|---|
| *min.* | *mm.* |
| 5 | 23 |
| 10 | 21 |
| 15 | 18 |
| 20 | 15 |
| 25 | 14 |
| 30 | 0 |

Diameter of inhibition zones of untreated extract was 30 mm.

decreased with increased temperatures, being completely destroyed after exposure to 100° C. for 30 minutes.

## SUMMARY

The thalli of 7 species of Aspergillus and 2 species of Penicillium were extracted with water, ether, and alcohol.

Of these preparations only the alcoholic extracts of *Penicillium notatum* and *Penicillium chrysogenum* contained materials having antibiotic activity for *Staphylococcus aureus-H*. The thalli of *Penicillium chrysogenum* yielded more potent extracts than did those of *Penicillium notatum*. The thalli removed from 9-day-old cultures yielded the most potent antibiotic material in both instances. The potency of the antibiotic from the thalli of both molds decreased somewhat with storage at 0° C. over a period of 6 weeks. Antibiotic activity was completely destroyed by heating to 100° C. for 30 minutes.

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List of articles from the laboratories of the Institutum Divi Thomae which have been published in other journals since the last issue of *The Studies* appeared.

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178. Nutini, L. G., Kelly, T. A., and McDowell, M. A.: Effect of *Streptococcus pyogenes* extracts and filtrates on various bacteria. *J. Bact.* **53** (1947): 575.

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